DISSERTATIONES SCHOLA DOCTORALIS SCIENTIAE CIRCUMIECTALIS,
ALIMENTARIAE, BIOLOGICAE. UNIVERSITATIS HELSINKIENSIS

34/2015

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Terpene Analysis and Transcript Profiling of the Conifer Response to *Heterobasidion annosum s.l.* Infection and *Hylobius abietis* Feeding

DEPARTMENT OF FOREST SCIENCES
FACULTY OF AGRICULTURE AND FORESTRY
DOCTORAL PROGRAMME IN PLANT SCIENCES
UNIVERSITY OF HELSINKI

Department of Forest Sciences
Faculty of Agriculture and Forestry
and
Doctoral Programme in Plant Sciences (DPPS)

University of Helsinki

Terpene analysis and transcript profiling of the conifer response to *Heterobasidion annosum s.l.* infection and *Hylobius abietis* feeding

Susanna Keriö

ACADEMIC DISSERTATION

To be presented for public examination, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, in the lecture room LS7 (Latokartanonkaari 9, B-building) on December 11th 2015 at 12 o'clock.

Helsinki 2015

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ISSN 2342-5423 (Print)
ISSN 2342-5431 (Online)
ISBN 978-951-51-1781-6 (Paperback)
ISBN 978-951-51-1782-3 (PDF)
https://ethesis.helsinki.fi/
Hansaprint 2015

Cover: Mixed forest with Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) (left). Mycelium and conidiophores of a homokaryotic *Heterobasidion parviporum* isolate growing on phloem of an inoculated Norway spruce tree (upper middle). Large pine weevil (*Hylobius abietis*) feeding on a conifer sapling (upper right). Lesions produced by highly susceptible and less susceptible Norway spruce clones (lower middle) and Scots pine trees (lower right) in response to inoculation with homokaryotic isolates of *Heterobasidion spp.* On the background, a composition of heatmaps showing the expression of LRR-type proteins in Scots pines in response to *H. annosum* inoculation.

CONTENTS

ABBREVIATIONS

BLAST	basic local alignment search tool
C	Control tree
CMK	cytidyl-methyl-D-erythritol kinase
DE	differentially expressed gene
DI	differentially induced gene
DNA	deoxyribonucleic acid
DXPS	deoxy-D-xylulose-5-phosphate synthase
ERF	ethylene responsive element binding factor
ET	ethylene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gbp	gigabasepair
GC-MS	Gas chromatography mass spectrometry
GGPPS	Geranylgeranyl-diphosphate synthase
GO	Gene ontology
GPPS	Geranyl-diphosphate synthase
GST	Glutathione S-transferases
D/H/M/PAMP	Damage/Herbivore/Microbe/Pathogen associated molecular pattern
HR	hypersensitive reponse
HS	Highly susceptible tree
IP	Invasion pattern
IPTR	Invasion pattern triggered response
JA	Jasmonic acid
LS	Less Susceptible tree
MAPK	mitogen activated protein kinase
Mbp	megabasepair
MeJA	methyljasmonate
MEP	Methylerythritol Phosphate Pathway
MeSA	methylsalicylate
NBS-LRR	Nucleotide binding site -Leucine rich repeat
PbTPS 3Car2	Pinus banksiana 3-carene synthase
PbTPS+aPin	Pinus banksiana +α-pinene synthase
PCD	Programmed cell death
PCR	polymerase chain reaction
PcTPS 3Car1	Pinus contorta 3-carene synthase
PcTPS-aPin	Pinus contorta -α-pinene synthase
PR	pathogenesis related proteins
PRR	pattern-recognition receptor
PsTPS5 far	Pinus sylvestris farnesene synthase

qPCR	quantitative polymerase chain reaction
QTL	quantitative trait loci
***R* gene**	major resistance gene
RNA	ribonucleic acid
RNAseq	RNA sequencing
ROS	reactive oxygen species
SA	salicylic acid
TAIR	The *Arabidopsis* Information Resource
TE	transposable element
TPS	terpene synthases
TPS	Terpene synthase
W	Wounded tree
VOC	volatile organic compound

LIST OF ORIGINAL PUBLICATIONS AND SUBMITTED MANUSCRIPTS

I. Kovalchuk, A., **Keriö, S.**, Oghenekaro, A., Jaber, E., Raffaello, T., Asiegbu, F.O. (2013). Antimicrobial defenses and resistance of forest trees: challenges and perspectives in a genomic era. *Annual Review of Phytopathology*, Vol. 51: 221-244 (2013).

II. **Keriö, S.**, Niemi, S. M., Haapanen, M., Daniel, G., and Asiegbu, F.O. Infection of *Picea abies* clones with a homokaryotic isolate of *Heterobasidion parviporum* under field conditions. *Canadian Journal of Forest Research*, 45: 226-234 (2015).

III. **Keriö, S.**, Jaber, E., Gürkan, S., Hartikainen, S., Raffaello, T., Kovalchuk, Lorenz, W.W., Holopainen, J.K., Dean, J.F.D., and Asiegbu, F.O. Changes in transcript and terpene profiles of *Pinus sylvestris* trees inoculated with *Heterobasidion annosum s.s.* under field conditions. (Research paper, submitted)

IV. Kovalchuk, A., Raffaello, T., Jaber, E., **Keriö, S.**, Ghimire, R., Lorenz, W. W., Dean, J.F.D., Holopainen, J. K., and Asiegbu, F.O. Activation of defence pathways in Scots pine bark after feeding by pine weevil *(Hylobius abietis*). *BMC Genomics*, **16**:352 (2015).

Account on author contribution:

I. **SK** wrote sections of the manuscript, and participated in planning the manuscript contents together with other authors.

II. **SK** and SMN conducted the field work and processed the samples. **SK** and SMN analyzed the data, **SK** did the microscopy work and drafted the manuscript. GD contributed to the microscopy work, FOA and MH conceived the study and helped to draft the manuscript. FOA, MH and **SK** contributed to the experimental design.

III. **SK** and EJ conducted the field work, processed the samples, isolated RNA, and performed the cDNA synthesis. GS and SH performed the terpene analysis. TR and **SK** performed the qPCR analysis, WWL and JFDD generated the pine transcriptome and developed the assembly used for microarray platform. TR, AK and **SK** analyzed the data. **SK** and AK drafted the manuscript. FOA and JKH conceived the study and helped to draft the manuscript. FOA, JKH and SK contributed to the experimental design.

IV. RG performed the herbivory treatment and VOC analysis, EJ and **SK** carried out RNA isolation and cDNA synthesis, TR performed the qPCR analysis, WWL and JFDD generated the pine transcriptome and developed the assembly used for microarray platform, AK and TR analysed the data, AK drafted the manuscript, JKH and FOA conceived the study, and contributed to its design and coordination and helped to draft the manuscript.

ABSTRACT

During their life, conifer trees are exposed to numerous attacks by fungal pathogens and herbivorous insects. The *Heterobasidion annosum* Fr. (Bref) *sensu lato* (*s.l.*) species complex includes some of the most serious necrotrophic fungal pathogens of conifer forests both in Eurasia and North America. In the European coniferous forests, *Heterobasidion annosum sensu stricto* (*s.s.*) attacks Scots pine (*Pinus sylvestris*) roots, whereas *Heterobasidion parviporum* causes the majority of decay in Norway spruce (*Picea abies*) in Northern Europe, causing severe economic losses. Despite the extensive damages caused by *H. annosum s.l.*, little is known about the genetic determinants of host resistance. Another significant health problem concerning Scots pine is caused by *Hylobius abietis*, the large pine weevil, which can cause severe damages in newly established Scots pine stands. To fight off pathogens and pests, trees have developed a broad repertoire of protective mechanisms, including several defence reactions which are activated upon fungal infection and insect attack. However, these reactions have not been comprehensively studied in conifers.

The aim of this research project was to study the responses of mature Norway spruce and Scots pine trees with varying levels of sensitivity to *H. parviporum* and *H. annosum s.s.* inoculation. We also tested the ability of homokaryotic *H. parviporum* and *H. annosum s.s.* isolates to infect mature conifer hosts and their ability to elicit defensive responses. Furthermore, we aimed to study the responses of Scots pine to *Hylobius abietis* feeding. As Scots pine has not been so extensively studied compared to spruce, the the emphasis in this work was on the terpene profiles and transcriptomic responses of Scots pine to *H. annosum s.s.* and *H. abietis* challenge. The transcriptomic responses were studied by a customised *Pinus taeda* oligonucleotide microarray with 36.5K cDNA elements, which was designed based on the *P. taeda* transcriptome.

Based on lesion length, we identified Norway spruce clones and Scots pine trees with varying levels of susceptibility to *Heterobasidion* infection. The results also supported the idea that homokaryotic isolates of *Heterobasidion* can colonize mature conifer hosts, and evoke host defence responses under field conditions. Both insect feeding and fungal infection induced the production of terpenes in Scots pine. Despite the terpene induction, only few genes related to terpene synthesis were induced. We also observed that high accumulation of terpenes is not necessarily an effective protective mechanism, and that δ-3-carene is potentially associated with higher tolerance to *H. annosum* infection in Scots pine. Induction of genes related to biotic and abiotic stress responses indicated a wide transcriptomic reprogramming in response to wounding, fungal infection and weevil feeding. Induction of genes related to signal perception and defence responses was observed in the *H. annosum* inoculated trees, and several putative disease resistance proteins and receptors were induced especially in the trees less susceptible to *H. annosum* inoculation. The microarray data provided an important insight into the transcriptional response of conifer trees to pathogen infection and insect herbivory. Based on the results, in addition to the Scots pine δ-3-carene synthases, the genes encoding putative disease resistance proteins and receptors are promising candidates for further research on the Scots pine resistance to *H. annosum*.

1 INTRODUCTION

1.1 Challenges for global forestry in a changing world

Forests provide several ecosystem services, thus affecting both local and global climate, air quality, ground water formation and quality, and soil erosion. Forests also provide habitats for several species of animals and plants, and their existence significantly affects the global biodiversity (Prunier et al. 2015). Trees are also an important source of raw material for the global forest sector, and the value of round wood reaches a remarkable 100 billion US dollars annually (FAO 2010). Competing land use interests including production of food for the global population of 7.13 billion human beings (World Bank, 2014), production of fodder for the approximately 68 billion annual rotating population of livestock (FAOSTAT 2013), biofuel production, conservational requirements, and requirement for new construction sites creates pressure to make the management of forest resources as efficient as possible (White et al. 2014). Establishment of mono-species stands and clear-cutting make the forest management efficient, but have a negative impact on biodiversity, and also make the trees more vulnerable to attacks by insects and fungi. Intensive large-scale management of forests creates ecological niches not normally present in the native forest ecosystems, and can open the door for insect outbreaks and pathogen epidemics caused by native species (Ennos 2015). Additionally, the vigorous global trade involving movement of living plants creates the risk of introducing alien pathogen and insect species, thus posing a threat to the stability of native forest ecosystems, and the global conifer-based part of the forest sector (Pautasso et al. 2015; Ennos 2015).

Anthropogenic climate change and international trade as an entry route for exotic forest pathogens and pests are shaping the world's forests in unforeseen ways, as the tree populations do not have the time needed to adapt to the changing environment and mostly have only weak tolerance to the exotic pests and pathogens (Pautasso et al. 2015; Ennos 2015; Prunier et al. 2015; Dukes et al. 2009; Wingfield et al. 2015). However, the invasiveness of introduced pathogens may also be explained by traits related to higher transmission potential compared to the native pathogens. This might be the case for *Heterobasidion irregulare* (Underw.) Garbel. & Otrosina, which has been introduced from North America into Italy during World War II. It has been observed that *H. irregulare* has higher ability to colonize dead wood and to reproduce sexually compared to the European sibling species *Heterobasidion annosum* (Fr.) Bref. *sensu stricto* (*s.s.*). (Giordano et al. 2014), whereas the virulence of *H. irregulare* and *H. annosum s.s.* on North American and European pines is on the same level (Garbelotto et al. 2010). Examples of insect pests and fungal pathogens expanding their territory due to climate change include the increasingly severe outbreaks of mountain pine beetle [*Dendroctonus ponderosae* Hopkins] in British Colombia (Cudmore et al. 2010), and increasing occurrences of the fungus causing Dothistroma needle blight [*Dothistoma septosporum* (Dorog.) Morelet] in the Northern hemisphere (Woods et al. 2005). Introduction of exotic pathogens and pests can have devastating effects on native plant populations, including serious damages on five needle pines due to white pine blister rust (WPBR) [*Cronartium ribicola* J.C. Fisch.], the near-extinction of American chestnut [*Castanea dentata* (Marsh.) Borkh.] due to chestnut blight [Cryphonectria parasitica (Murrill) Barr] and American elm [*Ulmus americana* L.] due to Dutch elm disease, which is caused by three fungi (*Ophiostoma sp.*) and vectored by bark beetles (Gilbert 2002). *D. septosporum* is also an introduced pathogen in the Southern hemisphere, where it

infects exotic pine plantations (Woods et al. 2005). Pine wood nematode [*Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle] causes pine wilt disease (PWD), which is globally one of the most destructive pine diseases. The pine wood nematode has spread from North America to Asia and Southern Europe, and the losses reach around 400 million euros in China alone (Xu et al. 2013). More recent introductions of exotic pathogens due to human activities include *Hymenoscyphus fraxineus* (Kowalski) Baral *et al*. causing ash dieback in Europe, and *Phytophthora ramorum* Werres *et al.* infecting several tree species both in Europe and North America (Pautasso et al. 2015). Moreover, exotic symbioses of wood-boring insects and fungi have devastating effects both in managed and natural forests, and on fruit-bearing trees. In their new environment, previously saproxylic insect-fungus symbioses can switch to attacking and killing live trees instead. For example, in North America the invasion of redbay ambrosia beetle [*Xyleborus glabratus* Eichhoff] with its associated fungus *Raffaelea spp*. which infects members of Lauraceae, including redbay, and threatens not only the populations of native laurels but also avocado cultivation. Another example is the spread of *Hypocryphalus mangiferae* carrying *Ceratocystis* sp. through mango-growing areas in South America and Middle East (Hulcr and Dunn 2011). Also a tree species which is planted outside the native distribution range may be more vulnerable to the native fungus-insect symbioses. For example outside its native range in the Western US, eastern black walnut (*Juglans nigra* L.) is highly susceptible to the thousand cankers disease (TCD), whereas the native walnuts have higher tolerance against the disease (Kolarik et al. 2011). TCD is caused by the fungus [Geosmithia morbida Kolařík *et al*.] vectored by the walnut twig beetle [*Pityophthorus juglandis* Blackman].

Conifers (*Coniferales*) are widely distributed plants with 650 recognized species which are found under various ecoclimatic conditions around the world. They are keystone species in many forest ecosystems, thus having a profound impact on the functions of forest ecosystems (Prunier et al. 2015). Conifer health is critical for the stability of forest ecosystems and sustainability of silvicultural operations, which makes research on the factors affecting conifer health of high importance. Maintenance of genetic variation of trees at a sufficient level for resistance and climate adaptation, either via breeding programs or natural populations, is the best possible insurance for the global change (Ennos 2015). Especially if establishment of mono-specific plantations either with exotics, trees poorly adapted to the local climate, or trees with low natural occurrence becomes mainstream forestry, routine integration of genetic selection programs for disease and pest resistance into forestry will become necessary. Resistance breeding will not likely eliminate the pathogens and pests, but will help to keep disease levels acceptable, and stabilizes the silvicultural systems so that the trees have time to adapt to the changing conditions (Ennos 2015). At the same time, forest management strategies should be modified to improve consideration of the risks posed by disease epidemics and outbreaks, and prevention of the introduction and establishment of exotic pests and pathogens (Telford et al. 2015).

1.2 Fungal pathogens and insects affecting forest health

1.2.1 Impact of management practices

Coexistence of trees and their biotic stressors is a common characteristic in forest ecosystems where the diversity of landscape, species, age and the genetic makeup of both pathogens and their hosts prohibits the emergence of strong selection pressure for pathogens or pests to reproduce rapidly (Gilbert 2002; Ennos 2015). Also in managed forest ecosystems, a balance often prevails and the damages on the tree population remain small. Host susceptibility, virulence or damage-causing ability of the pathogen or pest, and environmental conditions are factors that determine the extent of damage caused by the biotic stressors (Francl 2001). In forestry, management practices influence significantly the health conditions of a stand. Planting genetically and phenotypically uniform trees at high density creates the chance for pests and pathogens to exploit this readily available resource, and abnormal weather conditions can boost the development of epidemics or make trees more vulnerable to biotic damages (Ennos 2015; Wingfield et al. 2015). In the case of Southern pines and fusiform rust [*Cronartium quercuum* f. sp. *fusiforme* Burds & Snow], fertilization, establishment of even-aged pine plantations, and fire suppression has increased the occurrence of certain oak species, which are the alternate hosts of the pathogen, resulting in fusiform rust epidemics (Sniezko et al. 2014). Large-scale planting of exotic species increases the risk of serious damages over the long term, as the diverse populations of native pests and pathogens have adaptive advantage over the genetically uniform introduced hosts (Ennos 2015). For eucalypts [*Eucalyptus* sp.] which are widely grown as exotics and are often clonally propagated, there are examples of pathogens and pests which have shifted host from native plant hosts to eucalypts, such as the causal agent of *Eucalyptus* rust [*Puccinia psidii* Winter]. Currently *P. psidii* is among the most feared pathogens of eucalypts (Wingfield et al. 2008), and it has also been introduced to Australia despite quarantine efforts (Wingfield et al. 2015). Establishment of a global forest pest and disease management program is demanding, as some of the poorest countries lack resources to implement sufficient level of biosecurity actions, which sets the level for the efficacy of any control system (Wingfield et al. 2015).

1.2.2 *Heterobasidion annosum s.l.*

Some of the most devastating pathogens of conifers in the Northern Hemisphere are the members of the *Heterobasidion annosum sensu lato* (*s.l.*) complex (Asiegbu et al. 2005; Garbelotto and Gonthier 2013; Niemelä and Korhonen 1998). Species of *H. annosum s.l.* are necrotrophic pathogens with varying host specificity which cause damages on forests in all growth stages. *Heterobasidion annosum sensu stricto* (*s.s.*) has a wide host range, covering both coniferous and broadleaved trees, but the main hosts are found in the genus *Pinus*. In Northern Europe, the main host of *H. annosum s.s.* is Scots pine [*Pinus sylvestris* L.]. *Heterobasidion parviporum* Niemelä & Korhonen is specialized to infect Norway spruce [*Picea abies* Karst.], but can also infect conifers from other genus. *Heterobasidion abietinum* Niemelä & Korhonen occurs in Central Europe and infects firs (*Abies sp.*). The North American species *H. irregulare* infects mainly pines, whereas *Heterobasidion occidentale* Otrosina & Garbel. has a broader host range including species of fir, spruce, and hemlock (Garbelotto and Gonthier 2013). The global economic losses caused by *H. annosum s.l.* in terms of stem decay, growth reduction and tree mortality exceed 790 million euros annually (Woodward et al. 1998). Eradication of *H. annosum s.l.* is probably

impossible once the fungus has been established in a stand, due to its life strategy varying between necrotrophy during host colonization and saprotrophy during decomposition of dead wood, enabling the fungus to survive over decades on the same site (Garbelotto and Gonthier 2013).

The primary infections of *H. annosum s.l.* are established by homokaryotic basidiospores, which land on fresh stump surfaces and wounds on trees. *H. annosum s.l.* is a pathogen which benefits from forest management, as freshly created stumps provide an easy access for the fungus in the root system. The basidiospores germinate, and mating occurs when the hyphae with compatible mating types merge (Korhonen 1978), forming heterokaryotic mycelium. Secondary infections occur through the vegetative growth of the mycelium that spreads to neighboring stumps and trees via root contacts (Redfern and Stenlid 1998; Stenlid and Redfern 1998). The mycelium of *H. annosum s.s.* and *H. parviporum* colonizing infected trees is mostly heterokaryotic (Korhonen and Piri 1994). Due to the absence or very low occurrence of homokaryotic *H. annosum s.s.* and *H. parviporum* genets in infected trees, it has been presumed that homokaryotic isolates are less efficient than heterokaryons in colonizing standing trees (Korhonen and Stenlid 1998; Korhonen and Piri 1994). However, homokaryotic *H. parviporum* isolates able to colonize and potentially kill living Norway spruce trees have been recently found (Vainio et al. 2015). Also homokaryotic isolates of *H. occidentale* in North America have been observed to have similar or higher virulence compared to heterokaryotic isolates (Garbelotto et al. 1999; Garbelotto et al. 1997). Moreover, upon artificial inoculation, homokaryotic isolates of *H. annosum s.s.* infect both Norway spruce and Scots pine (Dalman et al. 2013), suggesting that heterokaryosis is not a prerequisite for virulence for the species of *H. annosum s.l.*

The lifestyle of switching between saprotrophy during wood degradation and necrotrophy during host colonization typical for *H. annosum s.l.* is also reflected by the the patterns of gene expression. For now, the model genome sequence for the *H. annosum s.l.* is based on *H. irregulare* (Olson et al. 2012). The 33.6 million basepair (Mbp) *H. irregulare* genome is predicted to contain 11 464 genes distributed on 14 chromosomes. The abundance of genes involved in plant cell wall degradation and their expression during growth on wood, cellulose and lignin reflects the ability of *H. irregulare* to colonize conifer wood. However, during colonization of a living host, a shift from primary towards secondary metabolism occurs, and pathogen transcripts related to stress tolerance are highly expressed (Olson et al. 2012). In addition, the enrichment of transposable elements (TEs) and unique genes in the *H. irregulare* quantitative trait loci (QTLs) related to pathogenicity indicates that these genomic regions are evolving at a high evolutionary rate, which is characteristic for pathogens under selective pressure because of strong host defences (Olson et al. 2012). Based on genome wide association (GWA) studies, it seems that *H. annosum s.l.* genome has regions of generic virulence factors shared by the whole species complex, but also species-specific virulence factors, reflecting the differences in the host ranges and possibly in the infection strategies applied on different hosts (Dalman et al. 2013). Moreover, it has been shown that traits related to transmission potential, such as wood degradation and sexual reproduction, may differentiate species with similar host range such as *H. irregulare* and *H. annosum s.s.* (Giordano et al. 2014).

Several methods are used to control *H. annosum s.l.* infections. In areas heavily infested by *H. parviporum*, Norway spruce should be replaced by some other tree species, and in case of *H. annosum s.s.* infestation, it may be reasonable to replace Scots pine with broadleaved trees. However, selection of durable material is important as *H. annosum s.s.* can also affect broadleaved species such as birch (Lygis et al. 2004). In Northern Europe, winter time logging and use of suitable machinery or manual felling at sites with sensitive terrain are means to avoid damage to the residual stand and roots (Korhonen et al. 1998). In addition, the removal of logging residues and stumps from the forest reduces the amount of inoculum in the area (Garbelotto and Gonthier 2013). Establishment of new stump infections can be efficiently prevented by performing logging operations during winter, but also by applying stump treatment, which is especially efficient and important in stands where no infection is present. Stumps treated with urea have elevated pH due to the ammonium formation by the tree enzymes, making the stumps less suitable as a substrate for *H. annosum s.l.* (Pratt et al. 1998). Biological control is done by treating the stumps with a spore suspension of the saprotrophic fungus *Phlebiopsis gigantea* (Fr.) Jülich, which are available as commercial products in Europe. The biocontrol mechanism of *P. gigantea* is most likely based on the higher saprobic ability and faster rate of stump colonization compared to *H. annosum s.l.* (Holdenrieder and Greig 1998).

1.2.3 *Hylobius abietis*

The large pine weevil (*Hylobius abietis* L., *Coleoptera*: *Curculionidae*) is regarded as one of the most important pine pests occurring in Europe and Asia, causing damage and mortality of young seedlings. The weevil breeds predominantly in the bark of conifer roots. It is a typical 'silvicultural' pest of plantation forestry, as it benefits from the conditions created by clear cuts and occurs only at low density in natural habitats. High weevil populations develop on the abundant root-stumps left in the ground after clear-cuts, and the stumps remain suitable as breeding sites for even three years. The weevils locate the host plants by both visual and volatile cues (Björklund et al. 2005), whereas stump roots are located by volatile cues, of which monoterpenes are the most important (Leather et al. 1999). Females lay eggs in June, and feeding is most active immediately before and at the time of the breeding season. The larvae utilize the phloem of the stump roots as a source of nutrition, and the metamorphosis from a larva to an adult weevil takes 1–5 years depending on temperature and local climate. In August, new adult weevils emerge from roots of pine stumps, and start their lives lasting from two to three years. The weevils feed on the bark of conifer seedlings during whole summer season, causing high seedling mortality by damaging the bark of the main stem. Damages to the seedlings are usually highest two years after the clear cut, when the new young beetles emerge from the stump roots. In the absence of seedlings, the adult weevils feed on the bark of young branches of mature conifers, and feed on plant bark before moving below ground to hibernate in October (Leather et al. 1999).

Currently, the damages caused by *H. abietis* are reduced by silvicultural methods or chemical treatment aiming at plant protection rather than pest control. Direct sewing, exposure of mineral soil surface around the seedlings, mounding, or fallow periods after the clear-cut can reduces the weevil damages. Interestingly, stumps treated with *P. gigantea* are not only protected against *H. annosum* infection, but are also less suitable as breeding material for the large pine weevil (Leather et al. 1999). Treatment of the seedlings by synthetic repelling chemicals or insecticides pre- or post-planting is the prominent plant

protection method due to its efficacy. However, because of environmental and health concerns in addition to the economic costs related to the use of repellent chemicals, alternative control methods are required (Långstrom and Day 2004; Leather et al. 1999). Environmentally friendly alternatives to synthetic deterrents include neem oil extracts (*Azadirachta indica*) (Thacker et al. 2003), and plant extracts which disturb the orientation and feeding behaviour of *H. abietis* (Egigu et al. 2011). In addition, pre-planting treatment of the seedlings with methyl jasmonate (MeJA), a volatile plant hormone central for triggering defences against herbivores, reduces girdling and mortality of pine seedlings over two growing seasons, which could also be an alternative for pesticide treatment (Zas et al. 2014). Some entomo-pathogenic fungi (EPF) and nematodes (EPN) are promising biocontrol agents. For example, stump treatment with an EPN [*Steinema carpocapsae* Weiser] significantly reduces the emergence of adult beetles for two years following the treatment, indicating potential as a biological agent that affect the *H. abietis* population size. Moreover, EPFs from *Beauveria sp.* show promise as fungi specifically targeting Coleoptera (Williams et al. 2013).

1.3 Defences of trees to fungal pathogens and insects

1.3.1 Constitutive and induced defences

Plants are constantly exposed to microbial stress and it is not a surprise that plants express a basal level of defence against a wide range of microbes. What makes the coexistence of trees, pathogenic fungi and insect pests even more intriguing is the difference in the lifespan of these organisms (Gilbert 2002). Conifer trees are extremely long-lived organisms unable to literally escape their enemies, which has obligated them to develop effective means to defend themselves against harmful microbes and herbivores over their whole lifespan. The longevity of trees sets requirements for the durability and resistance against harmful factors (Pearce 1996), but also for plasticity and capability to react to alterations in biotic and abiotic conditions (Gilbert 2002; Agrawal 2001). Repelling, defending, killing, and compartmentalization are the defensive events that a tree able to tolerate the attack will successfully go through (Franceschi et al. 2005; Telford et al. 2015). The development of the defensive state in a tree involves both general and specific defensive responses, and the strength of the response depends on the attacker and on the genetic toolbox the tree possess (Tobias and Guest 2014).

Protection of the living cells responsible for photosynthesis, water transport, cell division, and nutrient translocation is critical for the trees survival, and thus trees have evolved to be able to defend themselves against biotic and abiotic stress. The defence mechanisms can be divided into constitutive defences and inducible defences. Constitutive defences include structural and chemical barriers which are present irrespective of the attack (Jones and Dangl 2006). Leaves and needles are protected by a waxy cuticle, whereas the stem, branches, and roots of a tree are protected by bark. The highly suberized and lignified tissues of outer bark or rhytidome effectively protect the living cells in phloem, cambium, and sapwood. Bark also contains stone cells called sclereids, which slow down the chewing activities of insects (Franceschi et al. 2005). In younger trees, cortex protects the cambium until the secondary phloem develops. Lignified secondary cell walls also pose a challenge for the invading microbes and insects, as lignin is a complex polymer difficult to utilize as nutrition (Pearce 1996; Franceschi et al. 2005). In

addition, oleoresin rich in antimicrobial and antidigestive molecules is an important constitutive conifer defence present both in stem and needles. Inducible defences are activated following the recognition of the attacker (Jones and Dangl 2006). Inducible defences include the production of toxic or antimicrobial chemicals and proteins (PRs), programmed cell death (PCD), and formation of traumatic resin ducts and wound periderms. Most of PR proteins have apoplastic location, which makes them one of the first lines of defences encountered by the invaders (Van Loon et al. 2006). Chitinases and endoglucanases are PRs limiting fungal growth, whereas proteinase inhibitors are important for deterring insect herbivores (Howe and Jander 2008; Eyles et al. 2010). As a result of encountering a pathogen or a pest, the tree may develop induced resistance (IR), and have higher tolerance to subsequent attacks (Eyles et al. 2010; Franceschi et al. 2005). Induced defences may also prime the host and protect it from subsequent attacks. Priming occurs when plants ready their defenses in response to a signal or previous challenge for future attack. Primed defences are considered to be crucial to induced resistance, as they are metabolically cost-effective, and allow flexibility in the defences (Howe and Jander 2008).

1.3.2 Tripping the wire – How plants perceive the danger

As plants lack the adaptive antibody-driven immune responses of animals, the plants' defences rely on innate immunity, meaning that every living cell is responsible for the local initiation of defences (Zipfel 2014). The plant's ability to initialize defensive actions lies in their ability to recognize the invaders by receptor molecules The prevalent model used to describe the plant immune system is the Zigzag model (Figure 1a) (Jones and Dangl 2006), which has its merits in formally unifying the effects that general elicitors and specific virulence factors, or effectors, have on plant immunity, in a single model. In the Zigzag model, the plant immune system has components involved both in non-specific and specific recognition of pathogen-derived, non-self indicators. The basal, non-specific layer constitutes of pattern recognition receptors (PRRs), which are transmembrane receptors that recognize slowly evolving molecular structures or cues associated with the invader (Dodds and Rathjen 2010; Zipfel 2014). Microbe- or pathogen-associated molecular patterns (M/PAMPs) include for example chitin and β-glucan in fungal cell walls, or bacterial flagellin. However, certain MAMP epitopes are not recognized, which indicates that also the PRRs are dynamically evolving and probably do not provide durable resistance (Newman et al. 2013; Cook et al. 2015). MAMP-triggered immunity (MTI) is bypassed by pathogens which secrete effectors that cause perturbations in host defences, resulting in effector-triggered susceptibility (ETS) and enabling host colonization. The genes encoding effectors are often referred to as avirulence (*Avr*) genes and their products as Avr-proteins, as their recognition by the host may prevent the onset of disease. The more specific layer of defence based on effector recognition is driven by the activity of disease resistance (R) proteins, and effector recognition leads into effector-triggered immunity (ETI). Most R-proteins are intracellular receptor proteins with a nucleotide binding and a leucine rich repeat domain (NBS-LRRs) (Jones and Dangl 2006), which can either directly bind effectors, or guard another cellular target modified by effectors (Jones and Dangl 2006; Dodds and Rathjen 2010).

Despite its elegance, the Zigzag model has some shortfalls and has received criticism (Cook et al. 2015; Heil and Land 2014). The Zigzag model describes most accurately the interaction between plant hosts and biotrophic bacterial pathogens, and thus is not so suited to describe interactions of plants with necrotrophs, mutualists and insects. Also the dichotomy of stagnant versus evolving, MAMPs versus

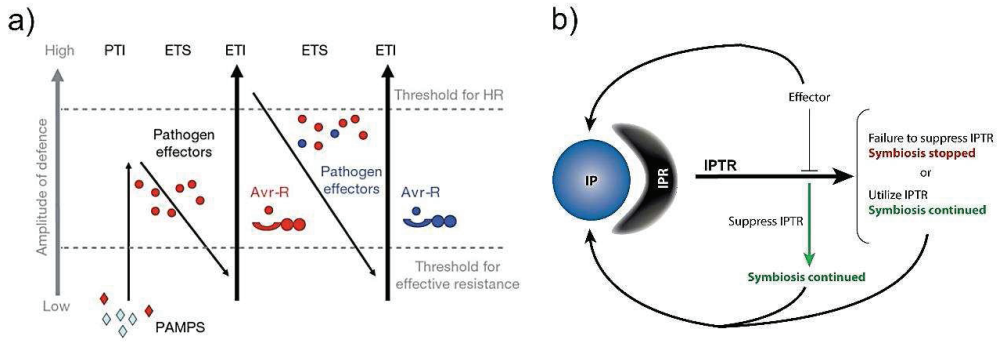

Figure 1. Perception of pathogens by the plant immune system according to the Zigzag model (a) (Jones and Dangl 2006), and perception of pathogens, insects, and host damage according to the Invasion Pattern model (b) (Cook et al. 2015).

effectors, PRRs versus R-proteins, and finally MTI versus ETI, moves the focus away from the dynamic and continuous nature of plant defences. The model also does not account for previous life history events, such as encounters with pathogens or insects (Cook et al. 2015), which is especially relevant for long-lived plants such as trees. Additionally, the Zigzag model does not cover damage-associated molecular patterns (DAMPs), which are plant-derived molecules that become targets of recognition only upon cell damage (Zipfel 2014; Heil and Land 2014). DAMPs allow the plants to recognize damaged-self based on endogenous signals, which is central especially for the perception of mechanical tissue damage, herbivore attack, and also necrotrophic pathogen invasion. DAMPs can be seen as the necessarybackground that prime the healthy and intact cells for correct recognition of MAMPs and effectors, and for a full immune response (Heil and Land 2014). As an alternative to the Zigzag model, an Invasion model (Figure 1b) has been suggested where the host perception of invasion patterns (IPs) is separated from the function of the molecules or processes that produce the IPs. Instead of D/M/PAMPs and effectors, in the Invasion model there are IPs that indicate invasion and give rise to signals of modified-self (Cook et al. 2015). The IPs are recognized by IP-receptors (IPRs) varying in their phylogenetic conservation and recognition specificity, leading to IP-triggered responses (IPTRs) which vary in strength and in the specificity of the signaling. IPTRs do not result into immunity by default, but either into the end or the continuation of invasion or infection, and invaders may use effectors to modify the IPTRs in their advantage. Moreover, the Invasion model recognizes that multiple defence responses are triggered simultaneously, and that host colonization or the IPTRs themselves can trigger subsequent responses (Cook et al. 2015).

The knowledge regarding the perception of insect herbivore damage by plants is limited, but it is presumed that the receptor-based recognition of herbivore-associated molecular patterns (HAMPs) acts in a similar way as in pathogen perception. It appears that plants use surveillance systems that involve both the perception of insect-derived HAMPs and monitoring of insect-damaged host targets (Howe and Jander 2008; Heil and Land 2014). Mechanical tissue damage is perceived as a DAMP which also induces several anti-insect defences, suggesting that endogenous signals, especially jasmonic acid (JA)

and volatile compounds, produced by the damaged cells are also important in amplifying the defence induction (Howe and Jander 2008; Heil and Land 2014). Mechanical damage is differentiated from herbivory most likely based on putative insect-derived HAMPs, which are found by hundreds for example in nematode saliva (Dodds and Rathjen 2010), but also in the oviposition fluid (Howe and Jander 2008). Volicitin is a fatty acid–amino acid conjugate (FAC) compound present in the saliva of larvae, and FACs in general cause alterations in the host proteome, transcriptome, and secondary metabolism (Howe and Jander 2008). Ascarosides are evolutionarily conserved signaling molecules that nematodes use to regulate development and social behavior (Manosalva et al. 2015). Even low concentrations of ascarosides are sufficient to enhance resistance to viral, bacterial, fungal, and oomycete pathogens in potato, tomato, barley, and *Arabidopsis*, suggesting that conserved insect-derived molecules may have practical applications in plant protection (Manosalva et al. 2015). Besides molecular triggers, also the spatial and temporal patterns of herbivory damage differentiate herbivory from mechanical damage, as sterile wounding causes only a non-detectable response (Howe and Jander 2008; Heil and Land 2014).

For many crop plants, several pairs of host R-proteins and their pathogen effector counterparts underlying the final result of plant-microbe interaction have been characterized, and the knowledge on the host R-proteins is also utilized in plant breeding to confer major gene resistance (Stergiopoulos and de Wit 2009; Dodds and Rathjen 2010). For conifers, their ability to distinguish fungal pathogens from mutualists and saprotrophs is indicated by distinct transcriptional (Adomas et al. 2008), histological (Sun et al. 2011; Asiegbu et al. 1999; Mucha et al. 2014), and hormonal and enzymatic (Likar and Regvar 2008) responses. However, at early time points after inoculation, there is considerable overlap in the transcript profiles of Norway spruce in response to *P. gigantea* and *H. annosum* (Arnerup et al. 2011; Arnerup et al. 2013), and in response to wounding and *H. annosum* (Lunden et al. 2015), possibly suggesting that colonization of host tissues is needed for activating more specific defences. In addition, there is evidence of the strong effect of a single gene in rendering a tree resistant to insect attack. The enhanced δ-3-carene accumulation and higher constitutive levels of δ-3-carene in Sitka spruce [*Picea sitchensis* (Bong.) Carr.] genotypes resistant to white pine weevil [*Pissodes strobi* Peck] are conferred by a *PsTPS-3car2* gene, which encodes a δ-3-carene synthase PsTPS-3car2 not found in susceptible genotypes (Hall et al. 2011). Moreover, compared to white spruce [*Picea glauca* (Voss.) Moench] genotypes susceptible to spruce budworm (SBW) [*Choristoneura fumiferana* (Clem)], resistant genotypes have higher transcript levels of *Pgβglu1*, which encodes for a corresponding β-glucosidase that affects the chemical composition of needles (Mageroy et al. 2015). Based on transcriptional profiling, β-glucosidases might also have importance in Norway spruce resistance to *H. annosum s.s.* (Lunden et al. 2015). An example of a documented R-gene controlled resistance in conifers is the resistance of several North American white pines to *C. ribicola*, the biotrophic pathogen causing WPBR. In WPBR-resistant white pines, the fungal growth is restricted to needles due to a hypersensitive (HR) like response controlled by a single major dominant gene (Sniezko et al. 2014). Also in Southern pines, R-genes conferring resistance to fusiform rust have been identified (Sniezko et al. 2014). However, in many cases the molecular counterparts involved in the interaction between trees and their pathogens and insect pests have not yet been identified. Based on transcriptomic studies, it seems that several genes affecting the ability of the tree tissues to resist necrotrophic pathogen invasion or insect damage

contribute to quantitative resistance. Quantitative resistance is caused by genes which contribute to the defences, but are not directly involved in recognizing the pathogen (Ennos 2015). In trees, resistance variation may be a result of several induced defences, genetic variation in several genes, pathogen or pest genotype, and of the influence of environmental conditions and tree age (Telford et al. 2015).

1.3.3 Transmission of the defence-related messages within plants

The signaling pathways involved in defence reactions are complex, contain both negative and positive feedback loops, and are partially overlapping (Meng and Zhang 2013). MAMP, DAMP or effector recognition triggers several early and ubiquitous defence responses, such as changes in calcium ion (Ca^{2+}) levels, production of reactive oxygen species (ROS), production of the gaseous plant hormone ethylene (ET), and activation of mitogen activated protein kinases (MAPKs) (Heil and Land 2014). These early defence events are needed to amplify the signals to activate various defence genes, synthesis of plant hormones, cell wall strengthening, production of toxic secondary metabolites and volatile compounds, PCD, HR, and induced resistance (IR) (Coll et al. 2011). It is presumed that the detection of the invader or damage creates a rapidly acting short-distance signal in the damaged tissues, which triggers MAPK cascades involved in perceiving environmental stress signals. Activation of the MAPK3/MAPK6 pathway is commonly reported in response to wounding. The calcium ion (Ca^{2+}) is implicated as a second messenger in many plant signaling pathways, and changes in Ca^{2+} levels are frequently detected in response to biotic stress. In *Arabidopsis*, Ca^{2+} influx triggered by external ATP perception induces ROS production, which in turn activates the MAPK3/MAPK6 (Meng and Zhang 2013; Heil and Land 2014). These damage-associated signals are mediated to the undamaged tissues by plant hormones, which act as systemic signals. Salicylic acid (SA) has important role in mediating the responses to biotrophic and hemibiotrophic pathogens, while jasmonic acid (JA) is triggered in response to herbivory, and a combination of JA and ET (JA/ET) is related to the defences against necrotrophic pathogens. MAPKs induced by plant hormones, the SA-induced protein kinase (SIPK), and wound-induced protein kinase (WIPK) which induces JA synthesis (Meng and Zhang 2013). SA together with ROS plays an important role in amplifying the signals to initialize HR to combat biotrophic pathogens (Coll et al. 2011). Due to their contrasting effects on resistance to pathogens with different lifestyles, SA and JA pathways are mostly antagonistic (Robert-Seilaniantz et al. 2011). In addition, abscisic acid (ABA), auxin, gibberellic acid (GA), and cytokinin (CK) affect the defence by regulating the hormonal balance. Virulent pathogens are able to modulate the plant hormonal signaling to promote host invasion (Robert-Seilaniantz et al. 2011).

SA is an important defence hormone that mediates both the local and distal signals related to biotrophic and hemibiotrophic pathogen defence through SA-responsive transcription factors and MAPKs (Robert-Seilaniantz et al. 2011). However, also non-chewing insects causing less severe damage trigger SA-mediated defences (Howe and Jander 2008). Together with ROS and Ca^{2+} accumulation, SA accumulation is required for the hypersensitive reaction (HR). SA is synthesized via two pathways, the phenylalanine ammonia lyase (PAL) pathway and the isochorismate synthase (ICS) pathway, and SA is used as a precursor for methyl salicylate (MeSA). PAL is also a key enzyme in the phenylpropanoid pathway, which is induced in response to wounding, fungal infection and insect attack (Ralph et al. 2006; Adomas et al. 2007; Kolosova 2010; Danielsson et al. 2011). SA synthesized via PAL has an important

role in regulating HR, whereas the SA synthesized by ICS is necessary for defence reactions against plant pathogens (Wildermuth et al. 2001). It has been observed that *H. annosum* inoculation triggers SA accumulation and increased PAL activity in Norway spruce seedlings, suggesting that *H. annosum* might benefit from the SA-controlled cell death for host colonization (Likar and Regvar 2008). In *Eucalyptus grandis* Hill ex Maiden, SA levels decrease in resistant genotypes as a response to infection with a necrotrophic pathogen [*Chrysoporthe austroafricana* Gryzehn. & M.J. Wingf.] (Mangwanda et al. 2015). A key component is NPR1 (NONEXPRESSOR of PR GENES 1), a positive transcription regulator of SA signaling. Upon presence of SA, NPR1 oligomers are degraded into functional NPR1 monomers which migrate from cytosol to nucleus, where they bind to TGA transcription factors, thus enhancing the TGA's binding to SA-responsive promoters and the expression of defence-related genes (Robert-Seilaniantz et al. 2011). SA is perceived by NPR3 and NPR4, which are paralogues of NPR1. NPR3 and NPR4 control NPR1 homeostasis in the cell by acting as adaptors that facilitate NPR1 degradation in response to SA (Fu et al. 2012). When NPR1 degradation is blocked in *Arabidopsis npr3 npr4* double mutants, also development of systemic acquired resistance (SAR) and PCD is blocked, which compromises the defences against biotrophic pathogens (Fu et al. 2012).

Jasmonates have a central role in mediating and regulating the defensive responses to herbivory and mechanical damage both locally and systemically. In addition to this, JA/ET are involved in resistance to necrotrophic pathogens and systemic transmission of defence signals, but also have roles in several aspects of plant development (Robert-Seilaniantz et al. 2011). Induction of defence-related genes, which are usually SA-mediated, as a response to *H. parviporum* inoculation seem to be primarily jasmonate-mediated in Norway spruce (Arnerup et al. 2013). Additionally, ET may play a role in mediating the signal of *H. annosum* attack into the distal tissues (Arnerup et al. 2013). Jasmonic acid (JA) is synthesized via the octadecanoid pathway, and JA is used as a precursor to synthesize the volatile signal molecule methyl jasmonate (MeJA), and jasmonoyl-isoleucine (Ja-Ile). Induction of the octadecanoid pathway as a response to insect feeding and wounding in Sitka spruce supports the importance of JA in coordinating the spruce defences to insects (Ralph et al. 2006). Moreover, exogenous MeJA induces terpene synthesis and traumatic resin duct production in conifers (Martin et al. 2002; Mumm et al. 2003), and increases resistance to *H. abietis* (Heijari et al. 2005; Zas et al. 2014) and to a blue-stain fungus [*Ceratocystis polonica* (Siem.) C. Moreau] (Zeneli et al. 2006). Impairment of the JA synthesis also compromises the anti-insect defences, which demonstrates the necessity of JA for defence against insects (Howe and Jander 2008). Jasmonates are detected by an F-box protein COI1. Jasmonates trigger interaction of COI1 with jasmonate zim-domain (JAZ) repressor proteins, resulting in JAZ degradation and de-repression of jasmonate-induced responses. However, transcripts of JAZ accumulate rapidly in response to wounding and fungal infection (Arnerup et al. 2013; Arnerup et al. 2011), indicating a negative feedback loop and a mechanism to restrain the costly jasmonate-induced defences upon cessations of the insect feeding and fungal attack (Howe and Jander 2008). There is also evidence that the feeding guild of the insect affects the activation of a pathway, as phloem-feeding insects that cause less damage possibly suppress JA-responsive genes and mostly activate SA-responsive genes. As jasmonate synthesis does not seem to be specific for herbivory responses, it has been proposed that the insect-derived effectors merely amplify the JA-dependent signals compared to mechanical damage alone,

creating a positive feedback loop. Also, herbivory-associated cues may act on distinct JAZ proteins that regulate herbivory-responsive genes (Howe and Jander 2008).

1.3.4 Role of secondary metabolites in tree defence

Trees, and plants in general, produce a wide array of secondary metabolites that have important roles in protecting the plant from various abiotic and biotic stress factors. Alkaloids, such as nicotine, caffeine, morphine, and cocaine, likely evolved as a defence against insect herbivory (Howe and Jander 2008). For conifers, terpenes form an important part of both the constitutive and induced chemical defences (Phillips and Croteau 1999; Eyles et al. 2010), and they form a very diverse class of secondary metabolites (Keeling and Bohlmann 2006). Terpenes constitute the oleoresin present in the resin canals, and due to the high volatility of monoterpenes, they are a major component of the volatile organic compound (VOC) emissions of conifers (Niinemets et al. 2013). In tree–insect herbivore interactions, the induction of chemical defences depends largely on the plant tissue, organ, insect feeding guild, and the insect species (Moreira et al. 2013; Köpke et al. 2010). In addition, timing of the chemical induction seems important for perceiving the signals of distress, as the volatile emissions attract insect parasitoids only within a limited time window, for example only at 72 hours after sawfly oviposition (Köpke et al. 2010; Köpke et al. 2008; Mumm et al. 2003).

Enhanced oleoresin production and induced terpene synthesis is a common response to both pathogen and insect attacks, and oleoresin functions both as a toxic chemical mixture and as a physical barrier (Eyles et al. 2010). Induction of terpenes as a response to challenge by fungi or insects has been observed in many contexts, and accumulation of terpenes and oleoresin has been reported to reduce the number of spruce bark beetle [*Ips typographus* L.] attacks in a dose-dependent manner (Zhao et al. 2011a), and to correlate with higher resistance to *C. polonica* in Norway spruce (Zeneli et al. 2006). Terpenes also have antimicrobial properties against fungal pathogens of trees *in vitro*, and many monoterpenes significantly slow the *in vitro* growth of several conifer-infecting fungi including *H. annosum s.l.* (Eckhardt et al. 2009; Schuck 1977; Zamponi et al. 2006; Kusumoto et al. 2014). In some instances, a strong link between resistance level and the chemotype has been established, thus making selection of more resistant forest regeneration material based on chemotype possible (Hall et al. 2011), demonstrating that specific genetic differences may underlie the chemotypic differences and the observed resistance phenotype. In Sitka spruce, increment in α-pinene proportion has been connected to lower susceptibility to *H. annosum s.s.* (Woodward et al. 2007) and to a blue-stain fungus *Ceratocystis polonica* Ciem. C. Moreau in Norway spruce (Zhao et al. 2011b). Moreover, resistance of slash pine [*Pinus elliottii* Engelm. var. *elliottii*] to fusiform rust correlates with higher constitutive β-phellandrene concentrations (Michelozzi et al. 1990; Michelozzi 1999), supporting the idea that chemical profiles have the potential to serve as selection guidelines for pathogen resistance.

Terpenes and other VOCs are emitted by plants both constitutively and in response to stress. Constitutive VOC emission rates are mainly driven by temperature and the volatility of the compound (Niinemets et al. 2013). When damaged, plants first release stored VOCs, followed by the release of *de novo* synthesized compounds. Aldehydes, alcohols, esters, and terpenes have high constitutive amounts in tissues, and upon damage their emission can produce a quick general signal for the receiving plants.

There against synthesis and emission of certain compounds, such as acyclic homoterpenes, specifically indicates herbivore feeding, and these compounds are referred to as herbivore-induced plant volatiles (HIPVs) (Holopainen and Blande 2013). The release of volatiles can deter further herbivore attacks, but also attract predators which associate plant-derived odours with the presence of the prey (Holopainen and Blande 2012; Holopainen and Blande 2013). For example, (*E*)-β-farnesene is a sesquiterpene commonly produced in response to herbivore attack to attract parasitoids (Howe and Jander 2008; Mumm et al. 2003). Exposure to VOCs causes transcriptional changes of defence related genes, accumulation of terpenes and MeJA, and may prime defences before the attacker is encountered. Volatile plant hormones (MeJA, MeSA, and ET) are important for mediating the responses to insect feeding, and often have synergistic effects with other VOCs. The metabolism of VOC production is well known, but it is fairly unclear how plants detect these signals, and how they differentiate conspecific signals, and signals from the same genotype. There is evidence that genetically identical plants perceive the volatile messages related to danger more strongly than genetically different plants and receive less damage, suggesting that mechanisms of self and non-self recognition cover volatile signals as well (Holopainen and Blande 2013). It has been observed that plants are able to adsorb VOCs and re-release them. It is possible that plants accumulate and enrich VOCs on their epidermis for more specific signal perception, and can also release them to distract insects. VOC signaling indicates that in future it is possible to use hypersensitive beacon plants which prime the defenses of other plants, and attract parasitoids to protect the whole plantation (Holopainen and Blande 2012).

1.4 Prospects for enhanced tree resistance

1.4.1 Deciphering the giant conifer genomes

Despite their economical and ecological importance, the research on conifer genomics lags far behind the research on angiosperm genomics. Conifer genomes are large and rich in repetitive sequences, which make genome assembly challenging. During the last few years, significant steps forward in conifer genome projects have been taken, in large part due to the development of next generation sequencing (NGS) technologies and the associated bioinformatics methods (Prunier et al. 2015; De La Torre et al. 2014). The giant 19-22 Gbp genomes of Norway spruce (*P. abies*) (Nystedt et al. 2013), Loblolly pine (*Pinus taeda* L.) (Neale et al. 2014; Wegrzyn et al. 2013), and white spruce (*Picea glauca*) (Birol et al. 2013) contain ca. 40 times the amount of DNA compared to genomes of angiosperm trees, such as western poplar (*Populus trichocarpa*) or eucalyptus (*E. grandis*) (Goodstein et al. 2012). Conifer genomes are rich in transposable elements (TEs) and long repeats, and the introns are much larger compared to *P. trichocarpa* or short-lived angiosperms (Neale et al. 2014). High level of synteny in gene order, largely conserved gene content between species, ancient origin of the genes, low level of variation in the chromosome number and size, and lack of genome duplication despite the large size of conifer genomes are unique features among seed plants, and indicate that conifer genomes are less variable and evolve slower compared to angiosperm genomes (Prunier et al. 2015; Leitch and Leitch 2012).

Regardless of their slow rate of evolution, conifers display a high degree of phenotypic plasticity in terms of occupying a wide range of ecological niches, and also when it comes to protective mechanisms. Plant *R* genes are known to undergo diversifying selection, which provides flexibility for pathogen detection.

However, the corresponding evolution to evade the defences is also going on in the pathogens (Ennos 2015). It seems that maintaining the *R*-gene diversity in tree genomes is one mechanism to compensate for the slower production of offspring compared to pathogens, as many trees have higher numbers of NBS-LRR genes (Tobias and Guest 2014) and genes encoding PR-proteins (Naidoo et al. 2014) compared to the genomes of short-lived perennials. Conifer genome and transcriptome analysis has revealed expansion of gene families related to secondary metabolism and defence, such as gymnosperm-specific cytochrome P450 and terpene synthases (TPS-d), which synthesize several mono-, di-, and sesquiterpene compounds present in oleoresin and volatile emissions. Most TPSs are multi-product enzymes with limited substrate specificity, and small changes in amino acid sequence change the product profiles (Keeling and Bohlmann 2006; Keeling et al. 2011; Roach et al. 2014; Keeling et al. 2008). In *P. glauca*, there are 83 unique TPSs with 28 putative pseudogenes, and 307 unique cytochrome P450s with 43 putative pseudogenes. The presence of pseudogenes suggests that these pathways are actively evolving, which reflects the importance of resin-based defences for the phenotypic plasticity and resilience of conifers (Warren et al. 2015).

The next challenges for the conifer genome projects include improving the contiguousness of the available genomes, which can be achieved by re-scaffolding the published genomes against existing transcriptome assemblies, linkage maps, and large-fragment sequences (Warren et al. 2015). Generation and analysis of this data is facilitated by the dropping prices of NGS and increasing computing power. Also sequencing of more genotypes will enable the development of genome-based selection methods for breeding purposes (De La Torre et al. 2014; Warren et al. 2015). Interesting directions for future conifer genomic research include studies on copy number variation and epigenetic changes (Prunier et al. 2015). The genome architecture may influence the flexibility of conifer defences, as the TEs abundantly present in tree genomes may regulate gene transcription *via* methylation patterns (Tobias and Guest 2014). For example in Scots pine, transcripts of TEs are induced in response to aphid feeding, heat stress, and SA treatment (Voronova et al. 2014). It is also possible that the small RNAs (sRNA) affecting the methylation status of transposable elements form an adaptive mechanism for conifer genomes. Mechanisms of gene expression regulation are an interesting target of study, as differences in the constitutive expression levels of the same gene may sometimes underpin the differences in the resistance phenotype (Warren et al. 2015).

1.4.2 Transcriptomics as a tool to study tree-pathogen and tree-insect interaction

Besides studying the genetic makeup of trees, the expression patterns of various genes reveal how the trees respond to various stimuli including pathogen infection and insect feeding. By studying the transcriptome of trees in response to biotic stress, the genes with potentially central role in tree defence can be identified. The transcriptomic responses of plants to fungal pathogens and insect pests have been extensively studied on several model species of angiosperms. However, only few comprehensive reports on the transcriptional responses of conifers to insect damage (Ralph et al. 2006; Kolosova 2010; Verne et al. 2011) or fungal pathogens (Danielsson et al. 2011; Lunden et al. 2015) have been published. The previous works used spruce species as their experimental models, and thus it would be beneficial to study the responses of other conifers to fungal infection and insect damage. The development of conifer-related

EST databases and microarrays (Lorenz et al. 2012; Kirst et al. 2003; Ralph et al. 2006; Ralph et al. 2008), and the progress in the sequencing projects of Norway spruce (Nystedt et al. 2013), white spruce (Birol et al. 2013; Warren et al. 2015) and Loblolly pine (Neale et al. 2014; Wegrzyn et al. 2013) genomes have increased the availability of genomic resources for spruce and pine.

Based on transcriptomic studies, it seems that several genes affecting the ability of the tree tissues to resist necrotrophic pathogen invasion or insect damage are induced in response to insect or pathogen challenge. The transcriptional response of mature Norway spruce roots to *H. annosum s.s.* infection has been studied by 454-sequencing (Danielsson et al. 2011) resulting in 14.36K isotigs, and of Scots pine seedlings to *H. annosum s.s.* infection by a microarray with 2.1K Loblolly pine ESTs (Adomas et al. 2007). Both studies reported the induction in the metabolic pathways involved in phenylpropanoid and flavonoid pathways. Induction of the phenylpropanoid and flavonoid pathways was also detected in Sitka spruce (Ralph et al. 2006) and in interior spruce [*Picea glauca x engelmannii*] (Kolosova 2010) in response to white pine weevil feeding, indicating overlap in reactions to pathogens and insects. Especially at early time points after inoculation, there is considerable overlap in the transcript profiles of Norway spruce to in response to fungal inoculation and wounding (Arnerup et al. 2011; Arnerup et al. 2013; Lunden et al. 2015). There may also be temporal variation in the defence activation in response to different threats, as the initiation of defences to insects seems to take longer compared to responses to fungal pathogens (Kolosova 2010).

The hunt for tree genes contributing to differences in the resistance phenotypes with the aid of transcriptome analysis can be done either by studying the constitutive transcriptome, or the induced transcriptome of trees with different resistance phenotypes. Studies describing the constitutive spruce transcriptome have reported only low numbers of differentially expressed genes between several genotypes of conifers either resistant or sensitive to insect damage (Verne et al. 2011; Mageroy et al. 2015). However, transcripts with high constitutive expression levels, such as white spruce *Pgβglu-1* in trees resistant to spruce budworm compared to susceptible trees, can contribute to constitutive defence, and can serve as putative markers of resistance (Mageroy et al. 2015). Interestingly, terpene and phenylpropanoid pathways are not constitutively expressed in interior spruce resistant to white pine weevil (Verne et al. 2011), suggesting that adequate pathway control is an important defensive trait, as unnecessary activation of defences in the absence of a threat may result in fitness costs (Ennos 2015). Also in response to pathogen or insect challenge, the transcript profiles cannot necessarily distinguish resistance phenotypes. In Norway spruce inoculated with *H. annosum s.s.*, the transcript levels related to the phenylpropanoid pathway are not very well correlated with the different resistance levels (Danielsson et al. 2011). *E. grandis* genotypes with differing resistance levels also display transcriptional overlap in their responses to *C. austroafricana*, but susceptible genotypes seem to have a delayed defence response, and are less efficient in modulating hormone signaling (Mangwanda et al. 2015). Japanese pines [*Pinus thunbergii* Parl.] susceptible to pine wood nematode (PWN) expressed SA- and JA/ET-responsive PR proteins from classes 1 – 6 more quickly and to a higher level compared to resistant pines, suggesting that defences mediated by these phytohormones are not very effective against PWN (Hirao et al. 2012). Moreover, PWN-resistant Japanese pines showed higher transcript diversity, and expressed peroxidases (e.g. PR9) and other genes related to cell wall fortification through oxidative reactions, indicating an

important role for cell wall resistance in tolerance to PWN. Additionally, CYP450s involved in terpene synthesis were up-regulated in resistant pines, linking oleoresin-based defences also to PWN resistance (Hirao et al. 2012). Similar responses to PWN infestation have been observed in Chinese red pine [*Pinus massoniana* Lamb.] (Xu et al. 2013). Many of the plant's defence reactions are activated upon fungal infection and insect attack, but the underlying regulatory mechanisms are not comprehensively studied yet, especially in conifer trees. Thus, better coverage in the microarray experiments might allow the detection of novel defence-relevant genes that might have escaped identification in the earlier experiments.

1.4.3 Breeding strategies aided by genomic selection

Tree improvement programs began in the 1950's, and they are currently an integral part of the operational silviculture programs around the world. Impressive results concerning volume yield, efficiency in the practical breeding work and techniques, and shortening of the breeding cycle for pines, eucalypts and other species have already been gained. Large-scale resistance breeding programs that resulted in operational planting of tested and improved material include the fusiform rust resistance in *P. taeda*, Dothistroma needle blight resistance in Monterey pine [*Pinus radiata* D. Don], WPBR resistance, and Sitka spruce durability to white pine weevil (Boshier and Buggs 2015). Requirements for increased yield per hectare and quick return of value, governmental subsidies for the use of renewable energy, and interest in more tailored wood material are important drivers for forest breeding programs. However, political decisions, changes in forest ownership structure, rapid technological development, and valuation of immaterial forest products cause uncertainty in the future wood markets, which also influences the future of forest breeding. Anthropogenic climate change, invasive exotic species, and competing land-use interests add up to the challenges that forest breeding is facing (White et al. 2014).

Because of long generational times of conifers, modern genetic and genomic techniques need to be integrated with conventional breeding (Harfouche et al. 2012). By combining genomics, transcriptomics, proteomics, metabolite profiling, and functional gene characterization, it is possible to characterize genes, anchor them on the gene space on the genome, and associate them with the respective biological function (De La Torre et al. 2014; Prunier et al. 2015; Harfouche et al. 2012). Knock-out and over-expression mutants can provide evidence of the role of the gene in question, and for example conifer cell cultures provide a homologous system that can be used to complement heterologous model systems (Duval et al. 2014). Studying how genes and pathways are connected is a powerful approach to identify key genes regulating several cascades, and to detect hubs that may be points of pathogen or pest manipulation. Advances in genomic technologies and reduction in sequencing costs make the assembly and sequencing of huge conifer genome feasible. For conifer breeding, it is important to improve the contiguousness of the available genomes, and also sequence more genotypes (De La Torre et al. 2014; Warren et al. 2015). Availability of high-quality genomes with high degree of gene annotation benefits the development of marker-based selection methods, for example for mate-pair allocation to increase gains (White et al. 2014). Also development of high-density marker maps has potential to identify genes of interest in terms of resistance breeding. A Norway spruce linkage map with 686 transcript-based SNP markers from 247 full-sib progenies was used to identify quantitative trait loci (QTLs) associated with resistance traits to *H. parviporum*. Altogether 13 QTLs associated with resistance traits were identified,

and four genes within the QTLs were involved in the phenylpropanoid and flavonoid pathways, which provides a valuable starting point for developing screening strategies (Lind et al. 2014). Information on putative resistance markers can be utilized in genomic selection (GS), where all markers contributing to a trait are combined to develop a model that is used to predict the genomic breeding value of progeny in future generations. When existing genomic information is combined together with the extensive field performance data from conventional forest breeding programs, it is possible to develop reliable GS models that can significantly save time, increase the efficiency of field testing, and shorten the breeding cycle by several years (Harfouche et al. 2012). GS suit well for the breeding of traits with low heritability, which is often the case with disease resistance. Moreover, GS can be used to improve mate-pair allocation and to aid in rapid decision-making to produce trees that have high adaptability to changing environmental conditions (Harfouche et al. 2012).

Although tree breeding programs are expected to move away from phenotypic selection to genomic selection, evaluation of heritability and durability of resistance traits based on phenotypic variation will still form the basis for marker association (Ennos 2015; Telford et al. 2015). As breeding and testing of the tree material is a long process, the core breeding strategy should be robust to the changes. Besides the central target of yield improvement through selection for better growth, health, and adaptability, it is recommended that forest breeding programs maintain adaptability, and embrace technology at all stages and scales to benefit from new advances (Harfouche et al. 2012). Moreover, inter- and transdisciplinary cooperation of scientists increases the pace of connecting genomic variation to phenotypic variation at the tree, stand, and landscape levels (White et al. 2014; Boshier and Buggs 2015)

2 OBJECTIVES AND HYPOTHESES

Despite the extensive damages that *H. annosum s.l.* and *H. abietis* cause in the European conifer forests, the knowledge on the genetic abilities of the hosts to resist infection and damages caused by these biotic agents is limited.

The objectives for this study were:

1. To conduct a literature review on the current knowledge and understanding on tree defences and on the challenges and perspectives related to the research on the field (I).

2. To test the ability of homokaryotic *H. parviporum* and *H. annosum s.s.* isolates to infect mature Norway spruce and Scots pine trees under field conditions, and to elicit defensive responses (Paper II, III).

3. To identify Scots pine trees and Norway spruce clones with differing levels of susceptibility to *H. annosum s.s.* and *H. parviporum*, respectively (II, III).

4. To study the changes in terpene profiles of Scots pine in response to *H. annosum s.s.* and *H. abietis* challenge (III, IV).

5. To investigate Scots pine transcriptome profiles in response to *H. annosum s.s.* and *H. abietis* challenge (III, IV).

The hypotheses for this study were:

1. The defensive responses vary according to tree genotype.

2. Homokaryotic isolates of *H. annosum s.l.* are able to infect mature trees and evoke the tree's defences under field conditions.

3. Both *H. annosum s.s.* infection and *H. abietis* feeding trigger defensive reactions which are reflected by the transcriptome and terpene profiles.

4. Scots pine responses to *H. annosum s.s.* infection can be differentiated from the responses to mechanical wounding based on terpene and transcript profiles.

3 MATERIALS AND METHODS

The materials, methods, type of plant material and fungal strains used in this study are summarized in Tables 1 and 2, and described in detail in the articles and manuscripts included in this thesis. Therefore, only brief descriptions are given below.

3.1 Preparation of fungal inoculum (II and III)

For the inoculum, spruce or pine wood dowels (7 mm × 10 mm) were moistened with water (20 ml/200 dowels) and autoclaved in glass jars for 20 minutes. The sterile dowels were placed on 2% malt extract agar plates for 3 weeks to be pre-colonized by *H. annosum s.l.* Autoclaved dowels placed on sterile malt extract agar medium were used as a control for the mock inoculation.

3.2 Inoculations and sample harvesting (II and III)

The trees (diameter 13-37 cm and height 11-28 m) were wounded with a 10 mm puncher so that the rhytidome and phellem, including the cambium were removed. Wood dowels pre-colonized by *H. annosum s.l.* were then placed in the wounds and covered with Parafilm®. For wounded control trees, wounds were inoculated with uncolonized wood dowels. After four months, the stem and root samples were collected by cutting down the trees and detaching the roots from the stumps. The samples were placed in cardboard bags, and after transportation, were stored at -20°C until used.

3.3 Lesion measurements (II and III)

The periderm tissues were removed using a knife and a small axe, and the extent of necrotic lesions in the phloem and xylem were measured (II, Figure 1). Phloem and xylem tissue samples containing both necrotic and unaffected tissue were collected from each inoculation and stored at -20°C until used.

3.4 Validation of infection and detection of the pathogen in infected tissues (II)

The success of the infection and colonization of Norway spruce clones by *H. parviporum* was determined by a combination of two standard methods, either by re-isolating the viable pathogen (Swedjemark and Stenlid 1997) or by PCR amplification of ribosomal ITS region (Hantula and Vainio 2003) from the collected tissue samples which had been stored at -20°C for two years. In addition, the hyphal colonization in tissue samples from clones V330 and V375 was observed microscopically. To confirm that the isolated fungal strains were identical to the original strain, pairing tests were performed (Korhonen 1978). In addition, since homokaryotic isolates of *H. parviporum* may also produce clamps (Korhonen 1978), nuclear genotypes of the retrieved *H. parviporum* isolates were identified with a primer designed for the amplification of M13 nuclear minisatellite fingerprints in order to differentiate nuclei identity of *H. annosum* s.l. (Dai et al. 2002; Karlsson 1994; Stenlid et al. 1994).

3.5 Analysis of the monoterpene and sesquiterpene profiles (III and IV)

3.5.1 Mature Scots pine inoculated with *H. annosum s.s.* (III)

Phloem and xylem samples were analysed for their monoterpene and sesquiterpene profiles by gas chromatography coupled with mass spectrophotometry. Strips of phloem and xylem tissue were excised from stem and root samples and were homogenized with liquid nitrogen. Thereafter, the samples were extracted with *n*-hexane as described elsewhere (Manninen et al. 2002). The terpene extracts were analysed by gas chromatography-mass spectrometry (GC-MS) as described by (Sallas et al. 2003).

3.5.2 Scots pine seedlings exposed to *H. abietis* feeding (IV)

The VOC samples were collected from the stem bark surface of both the control and the *Hylobius* damaged seedlings by enclosing the stem section just below the *Hylobius*-damaged bark area in polyethylene terephthalate bags. We collected 4.5 L of VOC samples from all the control and the damaged seedlings. The VOC samples were analysed by GC-MS as described by (Ghimire et al. 2013).

3.6 Microarray (III and IV)

Phloem tissue was excised from stem samples (Figure 1 in III), and RNA was extracted from the samples as described elsewhere (Chang et al. 1993). The RNA from each sample was processed as described in (Raffaello et al. 2014). Briefly, purified total RNA (100 ng) was subjected to reverse transcription and amplification, purified, and 4 µg of cDNA from each sample was sent to NimbleGen (NimbleGen, Iceland) for the hybridisation on loblolly pine (*Pinus taeda* L.) customised microarray. The customised *P. taeda* microarray was designed based on *P. taeda* transcriptome assembly PtNewbler1 available from the Conifer DBMagic database (Lorenz et al. 2012). The raw microarray data was composed of 109,270 probes (three probes per gene model) while the filtered and normalized file was composed of 36,424 gene models with expression data.

3.7 qPCR validation (III and IV)

A total of 13 (III) and 17 (IV) genes were validated by standard quantitative PCR (qPCR) as described in (Raffaello and Asiegbu 2013). The primers for the qPCR were designed based on the sequences of *P. taeda* isotigs and contigs. However, a subset of the primer sequences were further verified by aligning the isotigs with the reads from sequenced *P. sylvestris* cDNA libraries by courtesy of Prof. Teemu Teeri (Department of Agricultural Sciences, University of Helsinki, Finland).

Table 1. Summary of the methods used in this study.

Materials and methods	Publications
Inoculation of trees with wood dowels colonized by *H. annosum s.l.*	II, III
gDNA isolation	II
PCR	II
RNA isolation	III, IV
cDNA synthesis	III, IV
Microscopy	II
Microarray preparation and data analysis	III, IV
Gas chromatography and mass spectrometry	III, IV
Primer design	III, IV
Fungal isolates	II, III
Compatibility testing of fungal strains	II
Scots pine	III, IV
Field work	II, III, IV
Hylobius abietis	IV
Fungal genotyping	II

Table 2. Summary of the biological material used in the study.

Organism	Information	Publications
Picea abies[a]	Clones, age 40 years	II
Pinus sylvestris[b]	Seedlings, 6 years old	IV
Pinus sylvestris[c]	Mature, age 90-134 years	III
Heterobasidion annosum s.s.	Strain 03005, homokaryotic	III
Heterobasidion parviporum	Strain 01053, homokaryotic	II
Hylobius abietis		IV

[a]Study site: Former agricultural soil, Röykkä (60°30′N, 24°42′E, 100 m above sea level (a.s.l.)

[b]Study site: Research garden, Kuopio (62°53′N, 27°37′E, and 80 m a.s.l.)

[c]Study site: Drained peatland and Mineral soil site, Juupajoki (61°47′N; 24° 18′E; 150 m a.s.l.)

4 RESULTS AND DISCUSSION

4.1 Homokaryotic isolates of *H. annosum s.l.* evoke conifer defences under field conditions (II, III)

One of the aims in this study was to test the ability of homokaryotic *H. annosum s.s.* and *H. parviporum* isolates to infect mature conifer hosts under field conditions, and to elicit defensive responses. With the used artificial inoculation method, the homokaryotic isolates of *H. annosum s.s.* and *H. parviporum* used in the study were able to infect Scots pine and Norway spruce under field conditions. The fungi also evoked host defence responses, as could be deduced from the significant difference in the lesion size between the wounded and inoculated trees. The average length of necrosis in inoculated phloem was 2-3.5 times the length of the lesions in wounded phloem in Norway spruce and Scots pine (II, Table 2 and III, Figure 2). In II, it was demonstrated that the homokaryotic *H. parviporum* isolate infected 87% of the trees, and the homokaryotic state of the *H. parviporum* isolate was mostly maintained over the experimental period. In addition, microscopy observations on the colonization and penetration of host tissues by the studied homokaryotic *H. parviporum* isolate were similar to observations made with heterokaryotic isolates (Hietala et al. 2009; Johansson et al. 2004), indicating comparable colonization behaviour. However, the applied inoculation method does not correspond to the basidiospore-driven primary infections in nature, and the results do not clarify the epidemiological role of homokaryotic *H. annosum s.s.* and *H. parviporum* isolates. Moreover, only one isolate was used, although it would be interesting to compare the virulence of different homokaryons and artificially created heterokaryons. Therefore caution should be used when extrapolating the results to concern the potential of homokaryotic isolates for host infection and secondary spread within forest stands. On the other hand, homokaryotic isolates of *H. parviporum* have been recently reported to colonize living trees (Vainio et al. 2015), which could suggest that homokaryotic isolates of *H. annosum s.s.* and *H. parviporum* isolates play a role in host colonization and in evoking host defences. The study also provides further support for the use of homokaryotic *H. annosum s.l.* isolates in studies on the host-pathogen transcriptomics, as homokaryotic isolates are able to evoke defensive responses and infect the host trees. Reduced genetic variation due to the presence of only one copy of the genome in the haploid homokaryotic stage could provide more accurate estimates of the transcriptomic responses and gene regulation of the pathogen (Dalman et al. 2013).

4.2 Impact of tree genotype on the defensive responses (II, III)

In this study, lesion length was used to estimate the severity of symptoms in response to pathogen inoculation. A tree genotype that produces only a limited lesion suffers less quality damage, and is more likely to be able to limit the spreading of the fungus due to healing reactions (Swedjemark et al. 2001; Swedjemark and Karlsson 2006), which may effectively prevent the development of a disease. In accordance with earlier inoculation studies (Swedjemark and Karlsson 2004; Swedjemark et al. 1997), our results indicated differences in the responses of Norway spruce genotypes to inoculation with *H. parviporum* based on lesion length (II). We found the clone V330 (Novgorod, Northwest Russia) to be the least susceptible, while two half-sib clones, V374 and V375 (Pieksämäki, Central Finland), proved to be among the most susceptible for infection. In addition, the lesion length of the Scots pine trees as a response to *H. annosum s.s.* inoculation differed between the trees, whereas wounding did not cause

significant differences in the lesion lengths (III). For Norway spruce, the differences between clones were observed only based on root lesions (II). It is possible that the pathosystem in question affects the responses of hosts to pathogen inoculation, as the susceptibility of different host species to different *Heterobasidion* sp. varies (Garbelotto and Gonthier 2013). Also, in line with earlier studies reporting positive correlation between growth traits, lesion length and fungal growth (Swedjemark and Karlsson 2004; Arnerup et al. 2010), we found tree height and lesion length to be positively correlated both in Scots pine and Norway spruce (II, and Table 3). This might indicate that faster-growing trees could be more susceptible to mechanical damage and subsequent fungal infections, which further highlights the importance of good logging practices.

Table 3. Pearson correlation coefficients (*r*) relating Scots pine phloem lesion length to growth traits.

Lesion type	Dbh	Radial growth	Height	Living crown ratio	Age
Inoculated stem	0.44	0.69[a]	0.76[a]	-0.50	-0.72 [a]
Wounded stem	-0.31	-0.19	-0.25	-0.22	0.57

[a] Significant correlation at $P<0.05$

4.3 Terpene profiles of Scots pine phloem in response to *H. annosum s.s.* infection (III)

In addition to lesion size, the Scots pine trees with the smallest and largest lesions differed in terms of terpene concentrations. The absolute concentration of monoterpenes in the xylem samples from the highly susceptible trees was significantly higher compared to the other trees (III, Table 1). In general, fungal inoculation causes much higher terpene production compared to wounding alone (Fäldt et al. 2006; Woodward et al. 2007; Danielsson et al. 2011; Wallis et al. 2008). However, conifer genotypes representing different resistance phenotypes display highly varying terpene responses to abiotic and biotic stress (Zeneli et al. 2006). Additionally, it has also been observed that when Scots pine trees are inoculated with a non-virulent blue-stain fungus [*Ophistoma canum* (Münch) H. and P. Sydow], the terpene accumulation is much lower compared to trees inoculated with a virulent blue-stain fungus [*Leptographium wingfieldii* Morelet] (Fäldt et al. 2006). Also Sitka spruce genotypes less susceptible to *H. annosum* accumulate less terpenes compared to more susceptible genotypes (Woodward et al. 2007). However, higher terpene accumulation has also been reported to result in smaller lesions caused by *Diplodia pinea* (Desmaz.) J. Kick in Italian stone pine [Pinus pinea L.] (Bonello et al. 2008), and by *C. polonica* in Norway spruce (Zeneli et al. 2006). Moreover, in the case of insect attacks, high accumulation of terpenes protects the trees from insect damage (Raffa 2014). Thus, it is possible that the effect of terpene accumulation on pathogen resistance may vary according to the host-pathosystem in question.

The highly susceptible trees accumulated significantly higher amounts of terpenes compared to the less susceptible trees. The dominating monoterpene α-pinene had the highest proportions in the highly susceptible trees (III, Table 1). However, the less susceptible trees had significantly higher proportions of δ-3-carene compared to the highly susceptible trees, and δ-3-carene proportions had a significant

negative correlation with the lesion length (III, Table 2). The low proportion of δ-3-carene in the susceptible tree was also partially supported by the low expression levels of two δ-3-carene synthases based on qPCR analysis (III, Figure 7 and Figure 8). It is possible that the inoculated trees differed in their intrinsic capacity to produce and emit δ-3-carene and α-pinene (Bäck et al. 2012; Hiltunen 1975; Yazdani et al. 1985). The constitutive differences in δ-3-carene production could be explained by variation in the enzymatic capacity of the monoterpene synthases (Hall et al. 2011). The association of δ-3-carene and α-pinene with conifer resistance to fungal pathogens remains unclear. In spruce, increased amounts of α-pinene have been connected to lower susceptibility to fungal pathogens (Woodward et al. 2007; Zhao et al. 2011b), whereas increased δ-3-carene concentrations are associated with fungal infection (Zamponi et al. 2007; Danielsson et al. 2011; Zhao et al. 2011b). However, proportional increase in δ-3-carene content is higher in less susceptible than in susceptible Sitka spruce clones in response to *H. annosum s.s.* infectio (Woodward et al. 2007). Additionally, high δ-3-carene concentrations are connected to resistance to insects both in spruce (Hall et al. 2011) and in pine (Rocchini et al. 2000). To understand the putative functional role or association of δ-3-carene in the resistance of Scots pine to *H. annosum s.s.*, inventories in natural Scots pine populations, as well as isolation and characterization of the Scots pine δ-3-carene synthases merits further study.

4.4 Terpene emissions of Scots pine in response to pine weevil feeding (IV)

After eight days of *Hylobius* feeding on pine bark, the total emission of volatile organic compounds from the undamaged stem bark area were significantly increased when compared with the undamaged control plants (IV, Table 1), indicating a quantitative response to weevil feeding. The total monoterpenes emissions were only marginally significantly increased in the weevil-damaged plants. However, the observed increase in the emissions was measured from healthy bark below the damage area, and the magnitude of emission increment was comparable to bark emissions from damaged area (Heijari et al. 2011). This indicates a systemic response of the terpene synthesis to the bark damage which was earlier reported as increased terpene emissions from the needles of *Hylobius*-damaged pine (Heijari et al. 2011) and Norway spruce saplings (Blande et al. 2009). The emissions of δ-3-carene were significantly increased in response to the insect damage, which is in accordance with the observed association of δ-3-carene with the defensive responses of conifers to insect attack both in spruce (Hall et al. 2011) and in pine (Rocchini et al. 2000). Also emissions of limonene, total sesquiterpenes and several individual sesquiterpenes were significantly increased in the weevil-damaged plants. Six individual sesquiterpenes emitted by the *Hylobius*-damaged seedlings were not detected in the control plants. For example, the emission of *trans*-β-farnesene has repeatedly been shown to be induced by insect herbivory or oviposition (Mumm et al. 2003; Miller et al. 2005), and attraction of parasitoid and predatory insects (Mumm and Hilker 2005), thus indicating qualitative changes in the spectrum of compounds emitted by pine seedlings in response to *Hylobius* damage.

4.5 Scots pine transcript profiles in response to *H. annosum s.s.* inoculation and *Hylobius* feeding

4.5.1 Transcript profiles of Scots pine in response to *H. annosum s.s.* inoculation and *Hylobius* feeding

Both *H. annosum s.s.* inoculation and *H. abietis* feeding caused large-scale induction of gene expression compared to untreated control plants. We evidenced the activation of genes related to secondary metabolite synthesis, including genes related to the phenylpropanoid pathway, shikimate pathway, and phenylalanine biosynthesis pathway, as well as laccases, peroxidases, and dirigent proteins both in the three insect-damaged and the four *H. annosum s.s.* inoculated trees (Table 4), suggesting activation of processes related to cell wall fortification and secondary metabolism (Ferrer et al. 2008). One of the largest groups of genes induced in response to all the treatments were transcription factors, and both in the insect-damaged and *H. annosum s.s.* inoculated pines TFs showing similarity to ERF family (Singh et al. 2002) were highly expressed, which is in line with the central role of ethylene in responses of plants to mechanical damage, but also in plant interactions both with necrotrophs and herbivores (Glazebrook 2005; Howe and Jander 2008). Interestingly, genes related to the octadecanoid pathway central for JA synthesis had almost equal numbers of induced genes in wounded and insect-damaged samples, indicating the centrality of JA in responses to herbivory and mechanical damage. In the *H. annosum s.s.* inoculated trees, a large number of genes had high similarity to glutathione S-transferases, which have a role in detoxification and cell protection especially from oxidative stress (Sappl et al. 2009). As only few GSTs were induced in the insect-damaged Scots pine saplings, the induction of GSTs in response ro *H. annosum s.s.* could indicate stress responses to the accumulation of resin and other secondary metabolites in the affected tissues. Also high numbers of genes related to galactinol synthesis were induced, further suggesting protection from oxidative damage (Nishizawa et al. 2008). Finally, the largest number of receptor-like proteins in the LRR protein class was induced in the *H. annosum s.s.* inoculated trees, which could indicate specific responses to pathogen recognition and infection, as in the insect-damaged saplings the number of induced LRR genes was clearly lower (Table 4).

4.5.2 Differentially expressed genes in response to *Hylobius* feeding

The weevil feeding caused large-scale changes in the pine transcriptome. In total, 774 genes were significantly up-regulated more than 4-fold (IV). The simultaneous induction of a high number of genes emphasises that the defence against herbivores is a highly complex process involving numerous metabolic and signalling pathways and thus requiring a high degree of coordination between them. The biggest group of genes with similar functions among the top-25 up-regulated genes were putative protease and peptidase inhibitors with 9 representatives (IV, Table 2), emphasising the role of this class of proteins in the defence against herbivorous insects. It is assumed that they have an effect on the insect's digestive physiology by inhibiting gut proteases or disrupting the membranes of the gut epithelium (Harfouche et al. 2011). In addition, we observed up-regulation of genes involved in signal perception, signalling pathways, transcriptional regulation, plant hormone homeostasis (IV), secondary metabolism (IV, Figure 2 and 3) and defence responses (IV, Additional File 1: Table 1). Plant secondary metabolites play a central role in the constitutive and in the induced chemical defence against herbivores. In our experiment, we have documented a massive induction of genes involved in the different branches of the phenylpropanoid pathway (IV, Figure 2), as well as into some upstream steps and of genes

involved in the conversion of monolignols into lignin polymer. This finding once again emphasises the central role of this pathway in the plant defence response.

Table 4. Distribution of differentially expressed (Fold change > 2, $P < 0.05$ compared to control) genes into gene groups or pathways in response to wounding, *H. annosum s.s.* inoculation, and *H. abietis* feeding.

Pathway / Functional group	Induced Wounding	Induced *H. a.***	Induced *H. abietis* feeding	Repressed Wounding	Repressed *H. a.***	Repressed *H. abietis* feeding
Octadecanoid pathway	15	7	12	5	2	0
Phenylpropanoid pathway	16	13	4	6	1	0
Chalcone and stilbene synthases	6	3	5	0	0	0
Laccases	2	10	30	1	1	0
Class III peroxidases	19	15	20	0	0	0
Dirigent proteins	14	17	10	0	0	0
Terpenoid pathway	5	6	1	1	0	0
LRR receptors*	41	54	16	7	0	2
Transcription factors*	87	62	39	0	0	1
Protease inhibitors	12	15	18	0	0	0
beta-1,3-glucanases	19	14	15	0	0	1
Chitinases	15	6	15	0	0	0
Thaumatin-like proteins	4	4	3	0	0	0
Lipid-transfer proteins	4	5	5	0	0	1
Germin-like proteins	2	2	3	0	0	0
Glutathione S-transferases	35	70	3	0	0	0
Galactinol biosynthesis	58	61	14	0	0	0

*Only a subset of genes was analyzed for categories marked with an asterisk. The numbers for the whole microarray can be considerably higher.
***H. a.* = *H. annosum s. s.* inoculation

4.5.3 Transcriptional response of Scots pine to *H. annosum s.s.* infection and wounding

In the first part of our analysis, the sampled trees were divided into three groups: control group (C), wounded trees (W) and *H. annosum*-infected trees (H). The groups C and W included two trees each, whereas the group H consisted of four trees. Our analysis showed that both wounding and *H. annosum* infection caused substantial changes in the gene expression pattern of the studied Scots pine trees. We identified 2051 up-regulated genes and 2171 down-regulated genes in the infected trees (5.6% and 6.0%, respectively, of all genes represented on the microarray). The corresponding numbers for the wounded trees were 2239 up-regulated genes and 2631 down-regulated genes (6.1% and 7.2% of the microarray coverage, respectively). However, only 32% of up-regulated genes and 21% of down-regulated genes were unique for the infected trees (III, Figure 3). Among the 25 genes with the highest induction relative to control in the inoculated trees, we found eight genes with high similarity to galactinol synthase, eight genes without significat AT-hit, and two ERF transcription factors (III, Table 3). The abundance of

galactinol synthases among the highly induced genes could indicate important role for these genes in protecting the conifer tissues from oxidative stress during pathogen attack (Nishizawa et al. 2008). Additionally, among the genes which were specifically induced in the *H. annosum* inoculated trees, we found several genes with a putative function in plant defence responses or in the regulation of gene expression (III, Table 5). Notably, the list includes three genes with similarity to LRR receptors and three putative LRR receptor-like kinases. The function of those receptors deserves further investigation.

4.5.4 Activation of the phenylpropanoid pathway in response to *H. annosum* inoculation and insect feeding

The phenylpropanoid pathway plays a key role in plant defence reactions, and it is of pivotal importance for plant responses to microbial infections, insect attacks and mechanical damage. In this study, both wounding, fungal infection, and weevil feeding caused a massive induction of the genes with a predicted role in phenylpropanoid pathway (III, Figure 5; IV, Figure 2; Table 4). The phenylpropanoid pathway primarily supplies building blocks for the lignin biosynthesis, but also provides precursors for a number of important metabolites with antimicrobial and repellent activities, including flavonoids, anthocyanins, stilbenes, condensed tannins and phenolics (Ferrer et al. 2008). The small and water-soluble phenolic compounds produced via the phenylpropanoid pathway are essential for the trees since they represent a flexible mechanism of defence, and thus contribute positively to the resistance (Moreira et al. 2014). However, our results indicated that induction of the phenylpropanoid pathway was stronger in the HS trees compared to the LS trees (III, Table 4; Table 4). It has been observed that genes related to the phenylpropanoid and terpenoid pathways are not constitutively expressed in interior spruce resistant to white pine weevil (Verne et al. 2011). This suggests that adequate pathway control is an important defensive trait, since unnecessary activation of the defences in the absence of a threat may result in fitness costs (Ennos 2015). Timing of defence activation may also be critical, as for example less susceptible Norway spruce clones seem to accumulate catechin earlier compared to more susceptible genotypes (Danielsson et al. 2011).

4.5.5 Transcriptional responses of Scots pine trees representing different susceptibility levels to *H. annosum s.s.*

In the second part of the analysis on the response of Scots pine to *H. annosum s.s.* infection, we focused on the differentially induced genes (DIs) in the trees representing different resistance phenotypes. These trees are called either less susceptible (LS1, LS2) or highly susceptible (HS1, HS2). To study the differences between the inoculated trees representing different lesion phenotype in response to *H. annosum* inoculation, we conducted a GO over-representation analysis on the differentially induced genes (DIs) from the individual trees, which allowed a broad comparison of the processes occurring in the HS and LS trees. Terms related to signal transduction or signaling, stress responses and defence responses were over-represented only in the LS trees (III, Figure 4). Among these categories, there were 62 unique *Arabidopsis* GO hits which corresponded to 92 isotigs in the microarray. The 62 unique *Arabidopsis* GO hits included for example 4 ERFs, 10 disease resistance proteins, 14 receptor-like protein kinases, a dirigent protein, a peroxidase, a germin, and transmembrane receptors. Additionally, the 66 genes induced and shared only by the two LS trees (III, Figure 3c) included 15 isotigs showing

high similarity with disease resistance proteins and receptors, further suggesting differential responses for these trees to *H. annosum* inoculation. In contrast, among the 300 genes shared by the two HS trees (Figure 3c) there were only 13 isotigs with high similarity to disease resistance proteins and receptors. Over-representation of genes related to signal perception could indicate differential ability of the LS trees to respond to fungal invasion. However, it is possible that the over-representation in GO terms is biased by the use of the *Arabidopsis* as a reference genome instead of a tree genome, as tree genomes harbour proportionally high numbers of NBS-LRR class of *R*-genes (Tobias and Guest 2014). Additionally, the sample size in the study was rather limited, and the results should be validated on a larger number of trees. Still, the function of the identified genes deserves further investigation.

4.5.6 Transcript profiles of Scots pine terpene synthesis related genes in response to *H. annosum s.s.* inoculation and *Hylobius* feeding

Due to the central role of oleoresin-based defences for conifer health, we studied the expression patterns of transcripts related to terpene metabolism by cluster analysis. Despite the clear induction in terpene production both in response to pathogen infection and insect feeding, we did not find a clear transcriptional response in the terpene biosynthesis-related genes (III, Figure S2; IV, Figure 3). The only induced terpene synthase in the *Hylobius* damaged seedlings was a putative α-farnesene synthase (isotig17788). In the *H. annosum* inoculated trees, we observed induction in a few genes controlling the terpene precursor synthesis, especially in the initial and final catalytic steps of the MEP pathway. DXP synthase catalyses the first step in the MEP pathway which can play a rate-limiting role for the MEP-derived terpene production (Zulak and Bohlmann 2010; Zulak et al. 2009). The microarray results were successfully validated for 13 genes related to the terpene precursor and terpene synthesis (III, Figure 6), and the expression patterns were also studied by qPCR analysis for four infected, three wounded and two control trees (III, Figure S3). We compensated for the low number of hybridization probes for putative terpene synthases in the microarray by designing primers based on TPSs characterized in lodgepole pine (*Pinus contorta* Douglas), jack pine (*Pinus banksiana* Lamb.) (Hall et al. 2013), and for Scots pine sesquiterpene synthases (Köpke et al. 2010; Köpke et al. 2008). In accordance with elevated monoterpene concentrations in the highly susceptible trees, we observed up-regulation of putative α-pinene synthases (isotig17929 and isotig37123) compared to control, and the qPCR results for α-pinene synthases of *P. banksiana* (PbTPS-(+)apin) and *P. contorta* (PcTPS-(-)-apin) (Hall et al. 2013) gave similar results. As the microarray lacked any isotigs for δ-3-carene synthases and we found a significant difference in δ-3-carene concentration between the less susceptible and susceptible trees, we designed primers for δ-3-carene synthases of *P. banksiana* (PbTPS-3car2) and *P. contorta* (PcTPS-3car1) (Hall et al. 2013). We observed that the wounded trees had significantly higher expression levels for the δ-3-carene synthases compared to control. In addition, two susceptible trees had the lowest expression levels of PcTPS-3car1 compared to control, which could indicate division into high or low constitutive producers of δ-3-carene. As we also observed high transcript levels of terpene synthases in the wounded trees despite the comparably low concentration of terpenes, the results indicated that at least at later time points after inoculation, terpene concentrations are not well correlated with expression levels of terpene synthases.

As the expression patterns did not cluster clearly according to treatment and resistance phenotype, it is likely that genotypic effects influence greatly the expression of terpene synthases and genes related to the terpene precursor synthesis. The lack of correlation between the terpene concentrations and the respective TPSs in the mature Scots pine trees could also be explained by the sampling strategy. The induction of terpene synthesis would not necessarily be indicated by our data, because the peak in terpene synthase expression is typically reached within days after treatment (Zulak et al. 2009; Martin et al. 2002), and our samples were harvested after four months of wounding and *H. annosum s.s.* inoculation. Longer incubation time may be beneficial for differentiating the wound healing and pathogen inoculation, as at early time points, considerable overlap in the transcript responses of conifers to these stimuli has been reported (Arnerup et al. 2011; Arnerup et al. 2013; Lunden et al. 2015). As terpenes are metabolically expensive (Michelozzi 1999), terpene production will hardly be maintained for very long time after the initial damage. Additionally, as much of terpene synthesis occurs in the constitutive and traumatic resin ducts in xylem (Martin et al. 2002; Zulak and Bohlmann 2010), and thus the transcripts of xylem would not be accounted for in our phloem microarray analysis. Moreover, the deduced amino acid sequence of the encoded protein is not 100% identical to the sequences of the previously characterised enzymes, and it is known that even few amino acid changes can dramatically change the product spectrum of a terpene synthase (Keeling et al. 2008; Roach et al. 2014). Thus, the plasticity and sequence similarity of TPS genes may cause problems to qPCR validation and microarray hybridization for these genes. In subsequent transcriptome experiments with trees without a reference genome sequence, RNAseq would be a preferable choice to study the gene expression. Taken together with the low expression levels of terpene synthases contrasting with the high terpene concentrations in the *H. annosum s.s.* infected trees, these observations suggest that in young Scots pine majority of terpenes released from damaged tissue are synthesised elsewhere, whereas in the case of mature trees, existing storages of oleoresin contribute to the accumulation of resin near the damage site (Trapp and Croteau 2001).

5 SUMMARY AND CONCLUSIONS

In conclusion, we have identified Norway spruce clones and Scots pine trees that show different levels of susceptibility to *H. annosum s.l.* infection based on the development of necrotic lesions in stems and roots. The observed positive correlation between growth traits and lesion length might indicate that faster-growing trees can be more susceptible to mechanical damage and subsequent fungal infections. Furthermore, the results suggest that homokaryotic isolates of *H. annosum s.s.* and *H. parviporum* play a role in host colonization and in evoking host defences. The study also provides further support for the use of homokaryotic *H. annosum s.l.* isolates in studies on the host-pathogen transcriptome.

Both *H. annosum s.s.* inoculation and *H. abietis* feeding induced terpene production as measured by the terpene concentrations in the tissues and by VOC emissions, but induction of terpene synthesis at the transcriptome level was indicated only by a few genes. Also in Scots pine infected with *H. annosum s.s.*, induction of terpene synthases was not correlated with the terpene levels. The association of δ-3-carene concentration with the resistance level of Scots pine to *H. annosum s.s.* merits further study. These findings could indicate that in young Scots pine the majority of terpenes released from the damaged tissue are synthesised elsewhere and transported to the damage site, and in mature trees mainly the existing reserves of oleoresin contribute to the accumulation of terpenes.

The microarray data provided an important insight into the transcriptional response of conifer trees to insect herbivory and pathogen infection. Both *H. annosum* infection and *H. abietis* feeding caused induction in genes related to secondary metabolism, including genes related to the phenylpropanoid and terpene pathway. Additionally, in *H. annosum* inoculated trees, and especially in the highly susceptible trees, transcripts for GSTs were abundant, indicating high level of oxidative stress in the affected tissues. In contrast, we observed that transcripts related to signal transduction, stress responses, and defence responses were over-represented among the induced genes in the less susceptible trees. This could suggest that the trees that tolerate *H. annosum* infection are better at coordinating the defence responses, which was also indicated by the enrichment of transcripts with similarity to genes encoding receptors relevant for signal and pathogen perception. The the genes encoding putative disease resistance proteins and receptors are promising candidates for further research on the Scots pine resistance to *H. annosum*.

This study has provided insights into the defence mechanisms of Norway spruce and Scots pine against an economically destructive fungal pathogen and an insect pest. The applied microarray platform enabled wide analysis of the biological processes that take place in infected mature trees and in saplings under insect attack. Many of the genes identified in our experiment have been previously shown to be induced upon herbivore attack in other plant species, and their biological role is well-understood. At the same time, numerous induced and repressed genes could not be annotated, and those are particularly interesting as they might represent novel, previously uncharacterised components of the pine's defence machinery. They illustrate the massive changes in the host transcriptome upon fungal infection and insect attack. Many of the induced pathways are common between conifers and angiosperms, and the microarray platform will further facilitate the identification of diagnostic markers of defence responses with potential for conifer tree breeding.

6 FUTURE PERSPECTIVES

In this study, we applied short-term inoculation experiments to study the interaction of *H. annosum s.l.* and the conifer hosts. Short-term inoculation experiments may reveal conifer genotypes that are able to resist or contain the infection immediately after inoculation, but only long-term follow-up studies can demonstrate the field durability of the mature breeding material. Thus, a synthesis of field testing and parallel work on potentially transgenic or knock-out cell cultures, seed progenies and micropropagated clonal material could be a good strategy to identify genes that are critical for disease or insect durability. This would enable testing of the material with several fungal strains and challenging with several pest genotypes, which is not always possible in field experiments due to limited availability of plant material. Integration of the latest molecular methods into forest breeding on routine basis has the potential to provide robust information to support the design of models for genomic selection, and to tackle the challenges of breeding forest regeneration material with improved resistance to *H. annosum s.l.* and other pathogens and pests.

The results from this research project indicated an association between constitutively high δ-3-carene concentrations and higher resistance of Scots pine to *H. annosum s.s.* To study further the role or association of δ-3-carene in the pathosystem, inventories in natural Scots pine populations, as well as isolation and characterization of the Scots pine δ-3-carene and other monoterpene synthases merits further study. Combination of genomic, proteomic, and biochemical analysis would be needed to identify the Scots pine δ-3-carene synthase. For diagnostic purposes, availability of specific primers would be critical. Also, characterization of other terpene synthases of Scots pine in future studies should be of primary interest. Whether the terpene emission profiles of conifers could be used as indicators of degree of susceptibility could be tested by field inventories on infected sites, where analysis of volatile emissions would be combined with transcript profiling and genetic analysis of the δ-3-carene synthase. A potential sample pool could be the trees on the inventory plots of the Finnish National Forest Inventory (VMI).This could have innovation potential for increasing the accuracy of forest inventories and risk estimation in terms of forest health. Furthermore, the results of this project indicated that genes encoding receptor molecules involved in signaling and pathogen perception could underlie the higher tolerance of Scots pine to pathogenic fungi. These genes can function as a starting point for further studies aiming to characterize the mechanisms affecting the outcome of host-pathogen interaction.

The analysis of the pine transcriptional response to the insect herbivory and pathogen infection revealed massive induction of well-known genes, but also numerous genes that could not be annotated due to their low similarity to the known proteins, and those are particularly interesting as they might represent novel, previously uncharacterised components of the pine's defence machinery. To validate the role of the candidate genes in tree defence, transgenic conifer cell lines could be used to study the function of a specific gene. In subsequent studies focusing on conifer transcriptome, RNAseq could be applied to cover even larger part of the transcripts related to conifer defences. The obtained data are important for the large-scale comparative analysis of transcriptional responses to the fungal pathogens and herbivory in conifers and flowering plants, and the 36.4K gene pine microarray in this work is of importance for the identification and development of diagnostic markers for the resistance breeding of conifer trees.

ACKNOWLEDGEMENTS

The work included in this doctoral dissertation has been funded by the Academy of Finland, the Finnish Cultural Foundation, the Finnish Society of Forest Science, and by the Doctoral Program in Plant Sciences. The financial support is gratefully acknowledged. The work was conducted at the Department of Forest Sciences, and the support from the department and the University of Helsinki in the form of numerous travel grants is also gratefully acknowledged.

An essential reagent in the Ph.D. protocol is an inspiring and encouraging supervisor. Fred, since my bachelor studies, you have supported my academic development, and encouraged me to pursue a doctoral degree in the forest pathology (MPAT) lab. Your optimistic attitude, ambition, and enthusiasm about science and research have been truly inspiring. Without your encouragement, I would hardly had started my doctoral studies, and without your help and guidance, I would never have finished this thesis. Fred, I thank you for all the support during my years in the MPAT group, it has been a memorable experience.

My thesis follow-up group has also been of great help at setting the pace for the Ph.D. degree. Risto, Jarkko, and Harri, I learned to know you through my forestry studies, and I see you all as inspiring teachers and mentors. Matti, you got me the access to the clonal spruce material, and as a co-author also helped to improve one of the manuscripts. The annual meetings and discussions have been of great help, and I thank you all for the collaboration. The Doctoral Program in Plant Sciences and especially the coordinator, Karen, have also been a great support during the Ph.D. process. I also want to thank the pre-examiners of my thesis, Paolo Gonthier and Sanushka Naidoo, for their critical comments and revisions which helped to improve this thesis.

Without my awesome and hard-working co-authors, I would not have the amazing publications in my thesis. I have learned during my Ph.D. the significance of teamwork and importance of finding people who have the needed knowhow to make things happen. Andriy, Abbot, Emad, and Tommaso, you are precise and profound researchers who are always willing to help and discuss, and I have learned so much from all of you. Suvi, without you, I would still be peeling the logs in the workshop. Geoff, thank you for hosting me in your lab and introducing me to the fascinating world of SEM. Jarmo, Rajendra, Samuel, and Gürkan, thank you for the collaboration on the terpene analyses, and for getting me involved with the *Hylobius* part of the work. Jeff and Walt, thank you for the collaboration on the microarray aspects of the work. Thank you all, I deeply appreciate the time and effort you have invested in these projects.

During the years spent on the Ph.D. work, I have been lucky to have the best bunch of people in the MPAT group around me, who I also like to call my (academic) family. Without your support in the form of lab tips, protocols, help in the lab and in the field, lively scientific discussions, jokes, laughter, tears, great food, awesome conference trips, deep discussions about life, and wild parties, I would have lost my mind already years ago. I want to express my deepest gratitude to my wonderful colleagues and friends in the MPAT group: Eeva, Hongxin, Hui, Tommaso, Emad, Ximena, Abbot, Andriy, Riikka, Jiayao, Nicolas, Zhen, Mukrimin, Bikash, and Mengxia. Also former MPAT lab members and visiting researchers have influenced me with their awesome personalities both inside and outside the lab: Monica,

Julia, Jaeyong, Nevena, Erik, Lihua, Jinrong, Anna-Maija, Nina, and Chaowen. I have also had the chance to get to know Prof. Lee, one of the coolest academics I have ever met. In addition to the MPAT lab members, I have had the privilege to meet many great people from the labs of the Department of Agricultural Sciences: Marjo, Katrin, Milton, Mirko, Marcelina, Delfia, Tanja, Kean-Jin, Tuuli, Mikko, Jinhui, Iman, Bahram, Hany, and Xianbao. Also the people working on fungi and forest pathology in Luke Vantaa have enriched my Ph.D. life: Eeva, Sannakajsa, Juha, Rafiq, and Kashif.

Research is very much depending on the facilities, and when everything works well, you do not need to worry. Hyytiälä field station is the best possible site for field experiments, and I thank the amazing staff for their help. I also thank the forest engineers in Haapastensyrjä for their invaluable help with the sample harvesting. I am grateful to Marjut, who has helped me greatly during my Ph.D. studies with anything related to the lab. The administrative staff, including Jukka, Fatima, Sofia, and Leena, have taken care of the paperwork and I have been able to focus on science. Lab maintenance is also critical for the fluency of research. I have had many interesting discussion about lab equipment with Arto and Aarno, who try to make sure that we can mostly focus on research instead of the equipment. Also the lab technicians from the Department of Agricultural Sciences, Marja, Marjo, and Anu, have been very helpful both during my M.Sc. and Ph.D. Also thank you to all other supportive staff, you are all awesome!

My friends outside the lab have also played an important part in keeping me sane and in helping to forget about the work for a while. Although we do not meet so often, you are on my mind maybe more than you think. Thank you, Anna K., Anna P., Eeva, Essi, Johanna, Laura, Marina, Nea, Pinja, Terhi, and Viena, you are all lovely and dear to me! I also thank the spouses of my friends and colleagues, you are quite lovely too: Antti, Anders, Chao, Ilppo, Joni, Jukka, Kai, Kristian, Otto, Sascha, and Tero.

Last, but definitely not the least: my family. You have all helped me to forget about my work, but have also listened when I needed to talk. My parents, Raili and Pekka, you have always encouraged me and my sisters to study. Although you may not completely understand what exactly I have been doing for the last five years besides working long hours, you have never doubted that I could not make it. I thank you for all your support, you are the best! To my sisters, Kristiina and Annika, I thank you for cheering me up with your positive words and your crazy sense of humour at the hardest times, some things only sisters can understand. And thank you Lumi, my niece, for reminding me of what is important in life: shiny things, play, and candy. My gratitude extends also to my unofficial family-in-law: Kathy, Martti, Marika, and Marko. Thank you for believing in me, and for distracting me from work!

Finally, I thank my long-term companion Mikko. You have stood by my side in all the storms stirred by the Ph.D. side effects. You have also made efforts to distract me from the work, but have also let me finish, and even helped me with the field work. Without you, I would have lost my way a long time ago. I admire your patience, you are a remarkable person. As amazing as this journey towards the Ph.D. has been, I am so happy when I can say to you, “It is finally over, let there be life”.

Helsinki, November 2015

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Helsinki 2015 ISSN 2342-5423 ISBN 978-951-51-1781-6